REMARKS:

In this Amendment and Response, the currently pending claims are 119-195. Non-elected claims 15-42, 49-80, 82, 103, 105, 117, 118, drawn to a non-elected invention, are cancelled without prejudice or disclaimer. Claims 1-14, 43-48, 81, 83-102, 104 and 106-116 have been cancelled without prejudice or disclaimer and replaced by new claims 119-195 herein.

The Examiner's attention is drawn to the change in docket number for this application. Specifically, the docket number is changed from "2710-4007US1" to "PIT-010". It is respectfully requested that the new docket number be docketed for this application in the U.S. Patent and Trademark Office.

The currently pending claims are presented for the purpose of expediting the patent application process in accordance with the PTO's Patent Business Goals, 65 Fed. Reg. 54603 (September 8, 2000).

The new claims are fully supported by the application and claims as filed, and no new matter has been introduced into the application by virtue of the new claims. Specifically, support is found for the new claims in the instant specification, *inter alia*, on page 17, lines 2-8; on page 23, lines 13-19; and in Examples 1-4, pages 37-61, disclosing non-genetically engineered muscle-derived cells (MDCs) for the described repair and treatments. The instant disclosure supports MDCs in the absence of heterologous polynucleotide to effect the various treatments and repair functions as described for the present invention; such cells can be monitored following introduction

into a desired site for treatment by virtue of their carrying and expressing a marker gene, such as β-galactosidase, as described herein. In addition, MDCs carrying a heterologous polynucleotide allow for expression and production of the heterologous product at and around the site of introduction in a recipient host, as described and exemplified by MDCs carrying a vector containing a polynucleotide encoding inducible nitric oxide synthase (iNOS), which was expressed in measurable quantities following introduction of MDCs.

35 U.S.C. §112, first paragraph

Claims 1-14, 43-48, 81, 83-102, 104 and 106-116 have been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art ... to make and/or use the invention.

Applicants respectfully disagree with this rejection and submit that the claims as presented herein satisfy the requirements of §112, first paragraph, and address each of the Examiner's concerns as set forth on pages 4 and 5 of the October 24, 2002 office action.

The Examiner has opined on page 6 of the office action that "the specification fails to disclose a method of treating urinary stress incontinence by repairing any and all sites in the genitourinary tract tissue by injecting any and all muscle derived cells wherein the muscle derived cells are genetically engineered to express any and all bioactive molecules." It is submitted that the present claims more completely and

concisely define the subject matter elected for prosecution in the instant application and that the claims are commensurate in scope with the disclosure of the application.

The Examiner further refers to a publication of Young et al. who propose a strategy for treating stress incontinence by improving or curing detrusor instability. Applicants assume that the Examiner is referring to the paper of Suk Young Jung et al. (July, 1999, *J. Virol.*, 162:204-212) and point out that the publication date of this paper occurred after applicants' effective date of filing. Notwithstanding, applicants' specification teaches that stress urinary incontinence can be treated in accordance with the invention as presently claimed by introducing MDC into tissue of the genitourinary tract, for example, urethra or sphincter tissue.

Further examples of work from the inventors' laboratories, based on the teachings of the instant application and reported in the following publications and abstracts, demonstrate making and using the present invention without undue experimentation in accordance with the teachings of the specification. Representative publications in peer-reviewed forums serve to demonstrate that those in the pertinent field acknowledge and accept the use of MDC to affect repair of genitourinary tract injury or dysfunction, for example, as associated with urinary incontinence, in accordance with applicants' invention.

1) T. Yokoyama et al., 2001, "Persistence and Survival of Autologous Muscle Derived Cells Versus Bovine Collagen As Potential Treatment Of Stress Urinary Incontinence", *J. Urology*, 165:271-276.

2) T. Yokoyama et al., 2001, "Muscle-Derived Cell Transplantation and Differentiation Into Lower Urinary Tract Smooth Muscle", *Urology*, 57:826-831.

3) T. Yokoyama et al., 2001, "Autologous Primary Muscle-Derived Cells Transfer into the Lower Urinary Tract", *Tissue Engineering*, 7:395-404.

4) J. Lee et al., 2001, "New Functional Sphincter Formation After Allogenic Muscle Derived Stem Cell Injection into Denervated Rat Urethral Sphincter", *J. Urol. (Suppl.)*, 165:254, Abstract 1033.

The Examiner remarks that "urinary stress incontinence is not only caused by the weakening of sphincter muscle but is also the result of afferent nerve reflexes." (page 7 of the office action). The applicants respectfully disagree on this point. Those having skill in the art understand that urethral afferent nerve activity does not cause stress urinary incontinence. Rather, stress incontinence can induce or increase urethral afferent activity. Indeed, the activation of the urethral afferent nerve reflexes, caused by a weak sphincter muscle, can result in overactive bladder and urge incontinence. (See, S.Y. Jung et al., 1999, *J. Virol.*, 162:204-212, a copy of which is provided herewith). Moreover, the claimed invention provides a treatment for patients afflicted genitourinary tract injury or dysfunction associated with urinary incontinence, as well as for those having mixed incontinence, in which the stress urinary incontinence component of the affliction can be particularly treated.

It is respectfully submitted that the presently claimed invention involves the employment of applicants' described MDCs, which exhibit long-term survivability and myofiber formation in the muscle tissue of the genitourinary tract following injection, as shown by the examples in the instant specification. These surviving and proliferating

MDCs serve to bulk up and augment sphincter and bladder (e.g., bladder wall and detrusor) muscle tissue into which they are introduced. See, for example, Example 2, which describes the injection into the urethral wall to treat urethral injury in a mouse model system. Example 2 demonstrates that injected MDCs survive, as evidenced by being monitored for 30 to 60 days following introduction into the animal needing treatment, and allow the formation of regenerative myofibers in the urethral wall, which is comprised of muscle tissue. Animals that had been treated by MDC injection exhibited increased urethral pressure. Improved bladder contractility is also described in those animals which had injury to the urethra. Thus, the demonstrable formation of new muscle tissue following injection of MDC into injured tissue of the genitourinary tract, such as the urethral wall, supports the claimed invention as a treatment for repair and treatment of genitourinary tract injury, damage, or dysfunction.

Example 3 demonstrates that MDC injection into the bladder wall and the urethral wall modulates detrusor muscle contractility. In addition, nitric oxide (NO) was detected using a microsensor, showing release of NO from bladder wall area into which MDC that harbored polynucleotide encoding inducible nitric oxide synthase (iNOS) had been introduced. This result supports the ability of MDC to carry and express a heterologous polynucleotide encoding a bioactive molecule such as iNOS, to enhance the treatment process.

It is thus submitted that the present invention supports the repair of injured, damaged, or dysfunctional sphincter, bladder, or urethral muscle of the genitourinary tract following introduction of MDC into the relevant tissue. The invention relates also

to treatment of stress urinary incontinence following injection into genitourinary tract tissue, such as one or more of, for example, sphincter or urethra tissue. Applicants also point out that, as understood by the skilled practitioner in this art, bladder and urethra often serve as co-locations for therapies and functional studies, since urinary tract treatments can involve both the bladder and urethra. (See, e.g., T. Yamanishi et al., 2002, *J. Urol.*, 168:2706-2710; A. Madeiro et al., 2002, *Clin. Exp. Obst. & Gyn.*, XXIX:117-120)

In sum, MDC isolated from skeletal muscle can be employed as a nonallergenic and physiological agent to bulk up genitourinary tract muscle wall, thereby treating urinary incontinence, as well as enhancing coaptation and improving the damage or injury to urinary sphincter, bladder and urethral muscle tissues.

Applicants' submit that the claims as presented herein allow the skilled artisan at the time of the instant invention to make and use the invention as presently claimed without undue experimentation. Accordingly, withdrawal of the §112, first paragraph, rejection is respectfully requested.

35 U.S.C. §112, second paragraph

Claims 1-14, 43-48, 81, 83-102, 104 and 106-116 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite. The Examiner has indicated that the term "muscle derived cells" in the relevant claims is a relative term which renders the claims indefinite. Applicants traverse the rejection and submit that the claims as presented herein more particularly and distinctly describe the subject matter that is

regarded as the invention. Accordingly, withdrawal of the §112, second paragraph, rejection is respectfully requested.

AUTHORIZATION:

Should any fees, or additional fees, be deemed to be properly assessable during the pendancy of this application, or for the timely consideration of this Amendment and Response, the Commissioner is hereby authorized to charge any such additional fee(s), or to credit any overpayment, to Deposit Account No. 08-0219, Order No. PIT-010.

CONCLUSION:

It is believed that the instant application is in condition for allowance. An action progressing this application to issue is courteously urged. In the event that the Examiner is of the opinion that further discussion is necessary, the Examiner is hereby respectfully requested to telephone the applicant's undersigned representative at (212) 937-7315.

Respectfully submitted,

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URETHRAL AFFERENT NERVE ACTIVITY AFFECTS THE MICTURITION REFLEX; IMPLICATION FOR THE RELATIONSHIP BETWEEN STRESS INCONTINENCE AND DETRUSOR INSTABILITY

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ABSTRACT

Purpose: A causative relationship between stress urinary incontinence (SUI) and detrusor instability has been suspected but never proven. Many women with mixed incontinence have resolution of detrusor instability after surgical correction of SUI. We sought experimental support that stimulation of urethral afferent nerves can induce or change reflex detrusor contractions.

Materials and Methods: Urethral perfusion pressure and isovolumetric bladder pressure were measured with catheters inserted through the bladder dome in urethane anesthetized female S.D. rats (250 to 300 grams; n = 12). The catheter assembly was seated securely in the bladder neck to block passage of fluid between the bladder and urethra without affecting the nerve supply to the organs. The external urethra was not catheterized. Responses were examined in the control state at a urethral saline perfusion speed of 0.075 ml. per minute. Intraurethral drugs were administered following blockade of striated sphincter activity with intravenous α-bungarotoxin (0.1 mg./kg.).

Results: Stopping the urethral saline infusion caused a significant decrease in micturition frequency in approximately 50% of the animals studied (n = 12). Intraurethral lidocaine (1%) infused at 0.075 ml. per minute caused a slight decrease in urethral perfusion pressure but no change in detrusor contraction amplitude. However, intraurethral lidocaine caused a significant (45%) decrease in the bladder contraction frequency (n = 5). The micturition frequency returned to baseline 30 minutes after stopping lidocaine infusion. Intraurethral infusion of nitric oxide (NO) donors (S-nitroso-N-acetylpenicillamine [SNAP] (2 mM) or nitroprusside (1 mM) immediately decreased urethral perfusion pressure by 30 to 37% (n = 5). A 45 to 75% decrease (n = 5) in bladder contraction frequency was also seen, which was similar to that observed following lidocaine. Neither NO donor changed the amplitude of bladder contractions.

Conclusions: These results indicate that in the anesthetized rat activation of urethral afferents by urethral perfusion can modulate the micturition reflex. Thus in patients with stress urinary incontinence, leakage of urine into the proximal urethra may stimulate urethral afferents and facilitate voiding reflexes. This implies that stress incontinence can induce and/or increase detrusor instability. These findings have significant implications for the treatment of patients with mixed urge and stress incontinence. Correction of stress incontinence by surgery or pelvic floor exercise in patients with mixed incontinence may resolve the detrusor instability.

KEY WORDS: bladder, urethra, urodynamics, incontinence

A causative relationship between stress urinary incontinence (SUI) and detrusor instability has been suspected but never proven. Many women with mixed incontinence have resolution of detrusor instability after surgical correction of SUI while other women develop de novo detrusor instability after surgical correction of SUI.[1] We sought experimental support for the idea that stimulation of urethral afferent nerves, or nitric oxide, which may be involved in afferent neurotransmission,[2] can induce or change reflex detrusor contractions.

Approximately 50% of women with stress incontinence also complain of urinary frequency, urgency and/or urge incontinence.[3] In women with mixed stress and urge incontinence, successful surgical repair of SUI is associated with the cure of the urge incontinence in 50% to 75% of patients.[4] This large collective clinical experience among urologists and urogynecologists strongly supports a connection between urethral afferents and the micturition reflex.

During physiologic voiding, a drop in urethral pressure immediately precedes an elevation in bladder pressure, implicating urethral relaxation as the first part of micturition.[5] Pathologic fluctuations in urethral pressure (urethral instability) are a common finding in women with detrusor instability.[6] Urethral instability has been reported to occur in 42% of patients with detrusor instability and was strongly associated with the sequence of urethral relaxation prior to an unprovoked contraction.[7]

It has been suggested that unstable detrusor contractions may be initiated from the bladder outlet region, rather than from the detrusor itself.[8] The mechanisms underlying this hyperactivity are not known, but increased afferent activity, which may be produced by lack of an inhibitory substance in

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either the detrusor or the outlet region, has been suggested as one of the mechanisms for initiation of these contractions.[2]

Nitric oxide (NO) is the major neural inhibitory regulator of urethral tone in the rat and other species.[9,10] The NO-synthesizing enzyme, nitric oxide synthase (NOS), has been identified in nerve fibers of the detrusor, trigone and urethra, but most prominently in the smooth muscle region of the proximal urethra[2] and in pelvic plexus innervating the urethra.[11] Bennett et al have previously shown that reflex urethral smooth muscle relaxation in the anesthetized rat is mediated by NO.[10]

The primary goal of this study was to examine the effects of stimulation of urethral afferent nerves on reflex detrusor contraction. We believe this would have significant clinical relevance in unraveling the pathophysiology of mixed incontinence and defining the relationship between detrusor instability and urethral incompetence. In addition, we evaluated the effects of direct intraurethral administration of nitric oxide donors on urethral and bladder function.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing 250 gm. to 300 gm. were used in this study. All surgical and urodynamic procedures were performed under urethane anesthesia (1.2 gm./kg.; Sigma Chemical Co., St. Louis, Missouri). In each animal a cannula (PE-50) was placed in the carotid artery for monitoring blood pressure and in the jugular vein for intravenous drug administration. A tracheotomy tube was inserted to facilitate respiration and permit artificial ventilation following neuromuscular blockade.

To perform urodynamic measurement, the bladder and proximal urethra were exposed through a midline abdominal incision. To prevent the bladder from filling with urine during the experiment, both ureters were tied distally and cut, and the proximal ends were drained externally via gauze wicks. A cannula (PE-50) was inserted through the bladder dome, secured with a ligature, and used to fill or drain the bladder and monitor intravesical pressure. Urethral activity, measured as urethral perfusion pressure, was monitored using a double-lumen catheter (made of PE-160 and PE-50 with the tip embedded in a cone-shaped plug) which was introduced transvesically through a separate incision in the bladder dome and then seated securely in the bladder neck.[12,13] This preparation permitted functional separation of bladder and urethral activity without the risk of surgical damage to the vesicourethral innervation associated with a urethral ligation or total urethrotomy. The outer lumen of the catheter was connected to a pump for continuous saline infusion (0.075 ml. per minute) and the inner lumen was connected to a transducer for urethral pressure monitoring (fig. 1).

Experimental protocol. After a 30-minute postsurgical stabilization period, pressure recordings from the bladder and urethra were started. The bladder was filled with warm saline at a rate of 0.1 ml. per minute to induce reflex micturition, which was evident as rhythmic, large-amplitude bladder contractions. Then bladder filling was discontinued and isovolumetric pressure was recorded throughout the remainder of the experiment. The urethra was continuously infused with saline (0.075 ml. per minute) in an antegrade manner using a Harvard infusion pump. The saline infused into the urethra was allowed to drain freely through the urethral meatus. Thus isovolumetric bladder and urethral perfusion pressure were recorded independently and simultaneously. Urethral infusion was stopped to record the effects of infusion rate on bladder and urethral responses. Following this manipulation, the urethra was again infused with saline at the control rate (0.075 ml. per minute).

Administration of drugs. Drugs used in this study included α-bungarotoxin (0.1 mg./kg., intravenously (I.V.)), an irreversible blocker of striated muscle nicotinic receptors,[14] lidocaine

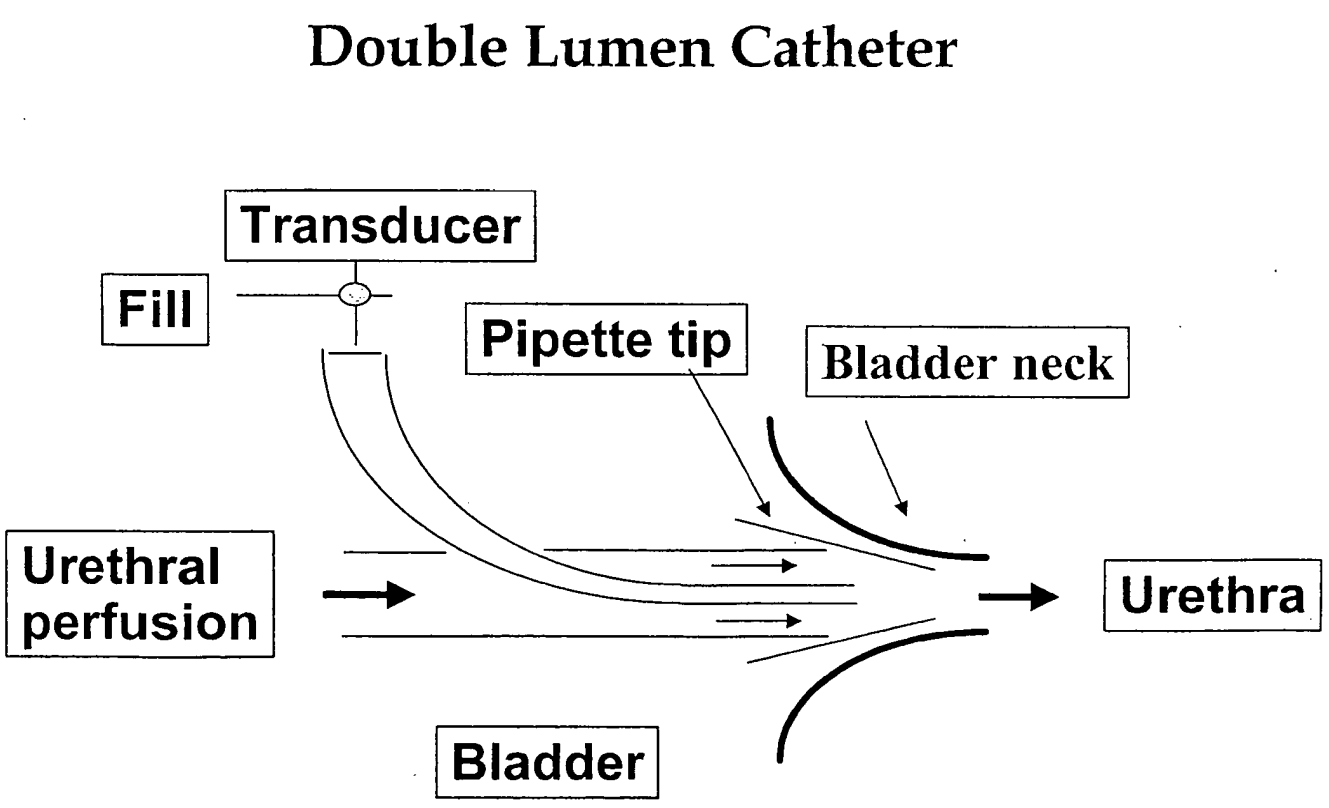

FIG. 1. Double lumen catheter used to separate bladder and urethra. Double lumen catheter, placed by separate cystostomy, to both perfuse urethra and also to record pressure in proximal urethra. Another catheter, not shown, is inserted by separate cystostomy, to record intravesical pressure.

(1%, intraurethral (I.U.)), S-nitroso-N-acetylpenicillamine (SNAP, 2 mM, I.U.), nitroprusside (1 mM, I.U.), N-nitro-L-arginine methyl ester (L-NAME, 20 mM, I.U.), and capsaicin (100 μM). The concentration of drugs chosen was based on prior research experience. 0.1 mg./kg. of α-bungarotoxin completely paralyzed the external urethral sphincter. The other drug dosages when given intravesically or intraurethrally were know to elicit maximum or near maximum bladder and urethra responses. SNAP was dissolved in saline with 1% DMSO and protected from light. Capsaicin was dissolved in 1% ethanol. All other drugs were dissolved in saline alone. Following α-bungarotoxin, all animals were artificially ventilated.

Statistical analysis. All data are reported as mean ± the standard error of the mean. Statistical evaluation was performed by the paired Student's *t* test. A value of $p < 0.05$ was reported as statistically significant.

RESULTS

The urethra exhibited coordinated reductions in pressure in association with large amplitude reflex bladder contractions. The urethral pressure wave during a micturition reflex in untreated animals was characterized by several components (fig. 2). The initial response was a slow drop in urethral pressure in conjunction with an early rise in intravesical pressure. A second response consisted of high frequency oscillations (HFOs) of the urethral pressure tracing that were superimposed on the period of urethral relaxation. The period of high frequency oscillations and urethral relaxation corresponded with the maximal amplitude of the bladder contractions. The period of urethral smooth muscle relaxation was followed by an increase in the urethral perfusion pressure to baseline or slightly above baseline for a brief period.

Effects of intraurethral saline infusion on urethral and bladder function. Stopping the intraurethral saline infusion during the control rate of infusion (0.075 ml. per minute) caused a significant decrease in bladder contraction frequency (from 0.40 ± 0.07 to 0.18 ± 0.15 per minute, $p = 0.04$; $n = 12$; fig. 2) in 50% of animals. However, the amplitudes of bladder contractions were not significantly changed (43.75 ± 1.19 versus 44.27 ± 0.64 cm. water). Bladder contraction frequency returned to control values after changing infusion rate into 0.075 ml. per minute.

Spontaneous rhythmic urethral activity between reflex bladder contractions was observed during infusion at 0.075 ml. per minute, but not observed when the infusion was stopped. Rhythmic urethral activity was not affected by intraurethral infusion of lidocaine (1%) or nitric oxide donors (see below) for 1 hour.

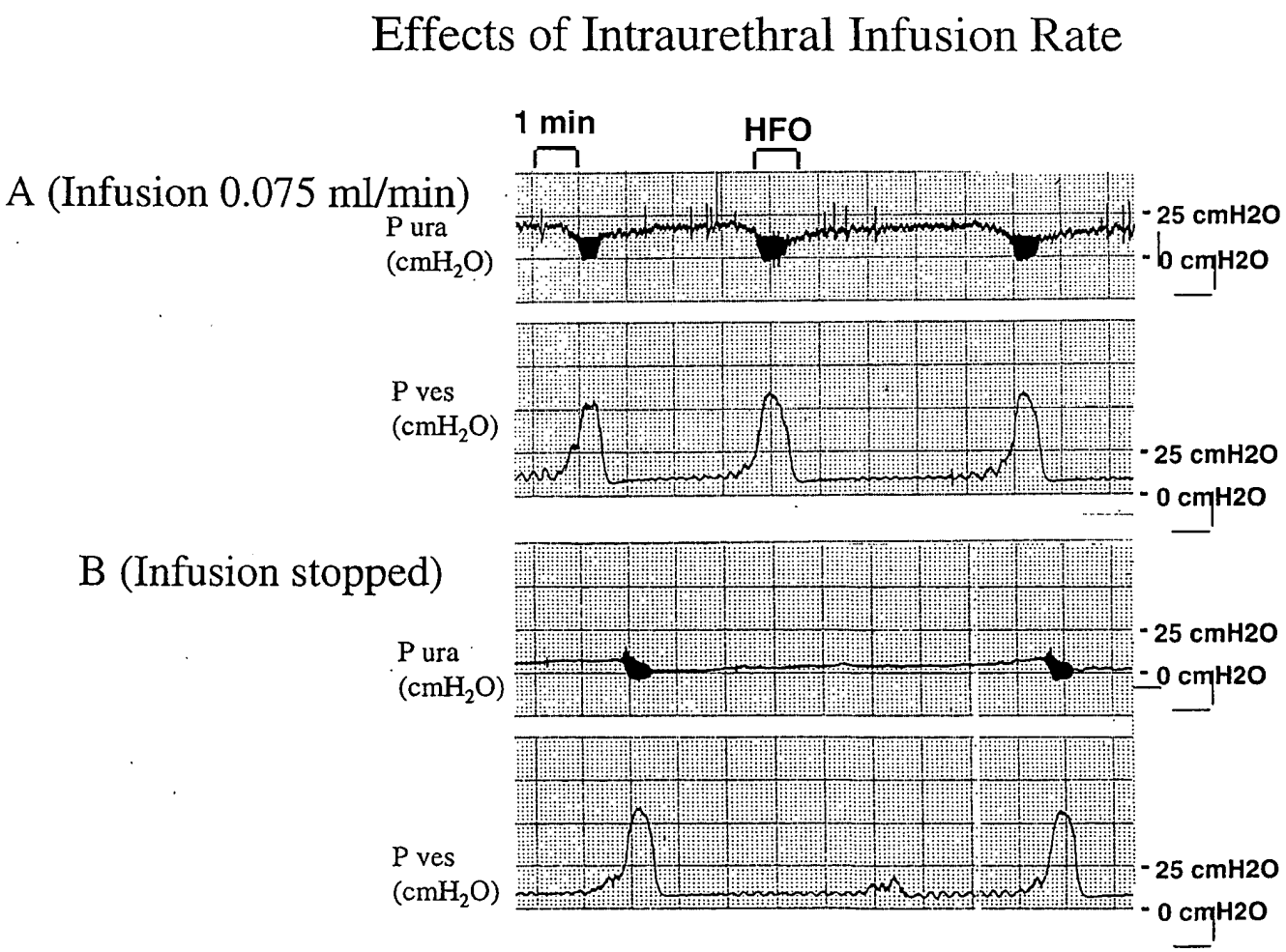

FIG. 2. Effects of intraurethral infusion flow rate on urethral and bladder pressure in normal female rats. *A*, in control state at urethral saline infusion speed of 0.075 ml. per minute, high frequency oscillations of urethral pressure (HFO) were recorded simultaneously during isovolumetric reflex bladder contractions. *B*, after stopping saline infusion bladder contraction frequency was significantly decreased, but amplitude of reflex bladder pressure was not changed. Spontaneous rhythmic urethral activity, observed during infusion at 0.075 ml. per minute, disappeared.

Neuromuscular blockade. Urethral smooth muscle function was examined after complete neuromuscular blockade with α-bungarotoxin (0.1 mg./kg., I.V.). This agent has a long duration of action, which allowed one dose to produce complete and selective neuromuscular blockade throughout the experiment (4 to 6 hours). After neuromuscular blockade, high frequency oscillations of urethral pressure were not observed during isovolumetric reflex bladder contractions (fig. 3). α-Bungarotoxin did not have an effect on bladder function. The urethral perfusion pressure between reflex bladder contractions (19.1 ± 1.26 cm. water; n = 10) was not significantly reduced by α-bungarotoxin, (18.3 ± 1.23 cm. water). The frequency of bladder contractions was not altered by α-bungarotoxin administration.

Effects of intraurethral lidocaine on urethral and bladder function. Intraurethral lidocaine (1%) infused at 0.075 ml. per minute significantly decreased the bladder contraction frequency by 45%, from 0.30 ± 0.02 to 0.14 ± 0.04 per minute (p = 0.001; n = 5) (fig. 3). Intraurethral lidocaine did not change urethral perfusion pressure (13.6 ± 1.10 to 12.5 ± 0.70 cm. water; p >0.05) and did not alter the amplitude of reflex bladder contractions (28.1 ± 2.17 to 29.4 ± 1.13 cm. water). The micturition frequency returned to baseline 30 minutes after stopping lidocaine infusion. Repeat lidocaine infusion resulted in a similar slowing of micturition frequencies.

Effects of intraurethral nitric oxide donors on urethral and bladder function. Intraurethral SNAP (2 mM, I.U.) immediately decreased urethral perfusion pressure from 18.3 ± 1.23 to 10.0 ±0.92 cm. water (p = 0.001; n = 5)(fig. 4). In addition, bladder contraction frequency was significantly decreased 45%–75% from 0.47 ± 0.04 to 0.33 ± 0.03 per minute (p = 0.001). The duration of reflex urethral relaxation was increased from 33.0 ± 11.7 to 92.0 ± 24.2 seconds (p = 0.001). The urethral relaxation persisted for 15 minutes after stopping SNAP infusion then returned to baseline.

Intraurethral nitroprusside (1 mM, I.U.) decreased the bladder contraction frequency from 0.67 ± 0.03 to 0.48 ± 0.07 per minute (p = 0.01; n = 5) and the interval between bladder contraction was more irregular. Neither SNAP nor nitroprusside significantly changed the amplitude of reflex bladder contractions (SNAP from 53.5 ± 1.29 to 52.7 ± 3.10 cm. water; nitroprusside from 43.2 ± 1.46 to 43.6 ± 1.80 cm.

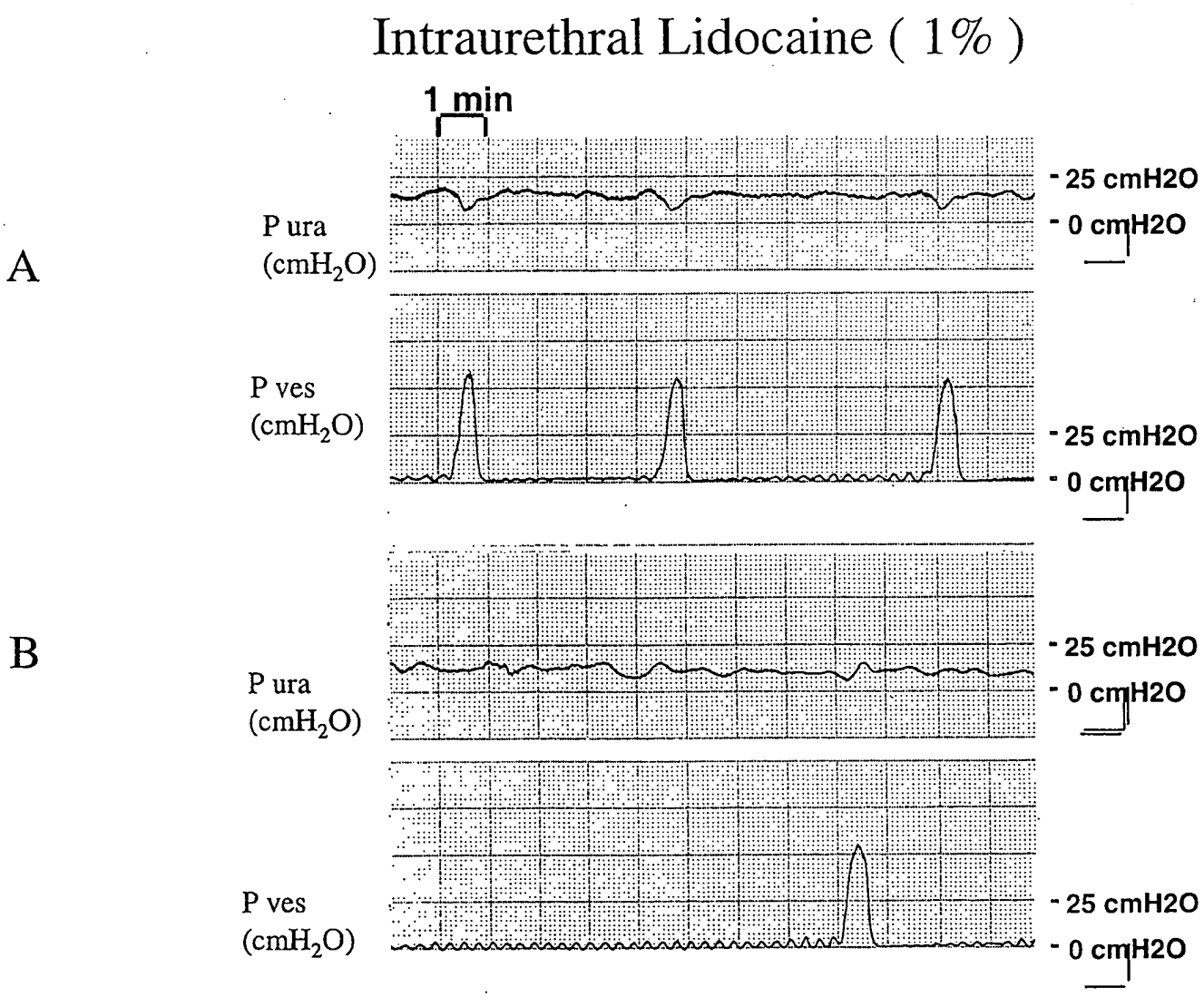

FIG. 3. Effects of intraurethral lidocaine on bladder and urethral pressure in normal female rats paralyzed with α- bungarotoxin (0.1 mg./kg., intravenously). *A*, before lidocaine treatment. *B*, after intraurethral administration of lidocaine (1%). Intraurethral lidocaine (1%) significantly decreased bladder contraction frequency by 45% but caused no change in urethral perfusion pressure and did not alter amplitude of reflex bladder contractions.

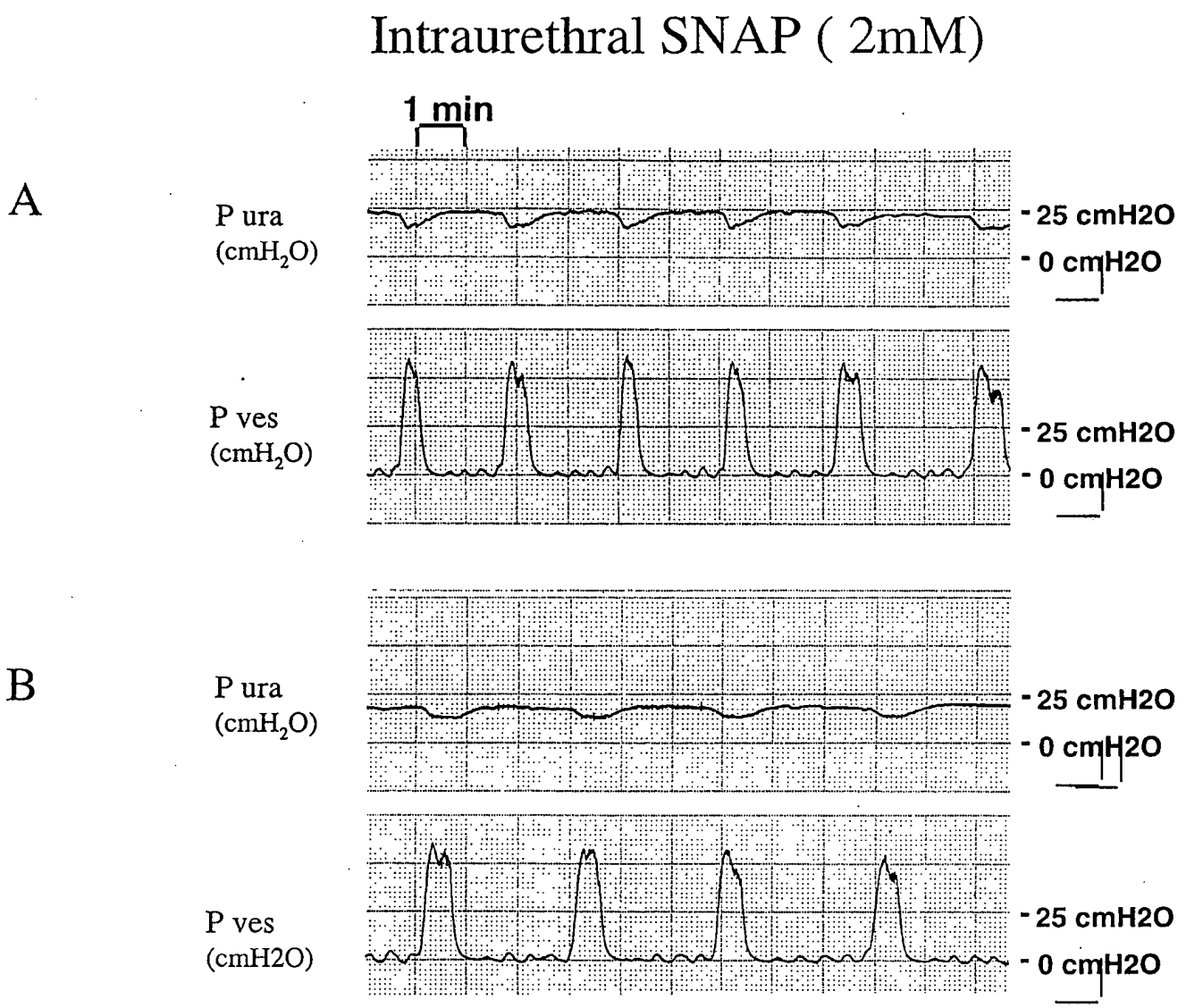

FIG. 4. Effects of intraurethral SNAP (2 mM) on bladder pressure and urethral pressure in normal female rats. *A*, before treatment. *B*, after intraurethral administration of SNAP. Urethral perfusion pressure immediately decreased. In addition, bladder contraction frequency was significantly decreased. Duration of reflex urethral relaxation was increased.

water). The blood pressure was not changed after intraurethral infusion of NO.

Effects of intraurethral NOS inhibitor on urethral and bladder function. Intraurethral administration of a NO synthase inhibitor (L-NAME, 20 mM, I.U.) did not significantly change urethral perfusion pressure (15.2 ± 1.09 to 14.3 ± 0.86 cm. water, n = 8)(fig. 5). L-NAME did not change the nadir urethral relaxation pressure from 5.52 ± 0.64 to 6.25 ± 1.85 cm. water (n = 8). Intraurethral administration of L-NAME did not have any effect on micturition frequency.

Effects of intraurethral capsaicin on urethral and bladder function. Intraurethral administration of 100 μM capsaicin, a neurotoxin that initially stimulates and then desensitizes unmyelinated afferent neurons,[15] elicited a biphasic change in bladder activity. Initially, within minutes after intraurethral capsaicin instillation the bladder contraction frequency increased from 0.40 ± 0.08 to 0.68 ± 0.16 per minute (n = 6)(p = 0.01) but within 15 to 30 minutes the bladder activity was completely blocked (fig. 6). Urethral relaxation responses disappeared but urethral perfusion pressure was unchanged after I.U. capsaicin administration.

DISCUSSION

The functions of the lower urinary tract to store and release urine depend on complex neural mechanisms that regulate the activity of the bladder and the various components of the urethral outlet.[16] Various reflex pathways have been identified[16–19] that are activated by bladder or urethral afferents and which can facilitate or inhibit voiding. During micturition, the nervous system regulates the muscles of the bladder base, proximal urethra and external urethral sphincter to induce urethral relaxation and promote urine flow. Barrington and Mahony et al have described micturition reflexes, some facilitatory and some inhibitory.[18,19] In the healthy adult, urine enters the urethra only during micturition.[20] However, urine is present in the urethra in patients with urinary incontinence. The entry of urine into the urethra could itself act as a stimulus to potentiate or even initiate detrusor hyperactivity.[21]

Urine or fluid in the posterior urethra may result in urgency to void and an involuntary bladder contraction.[22] Barrington reported that running water through the urethra or distention of the proximal urethra caused contraction of

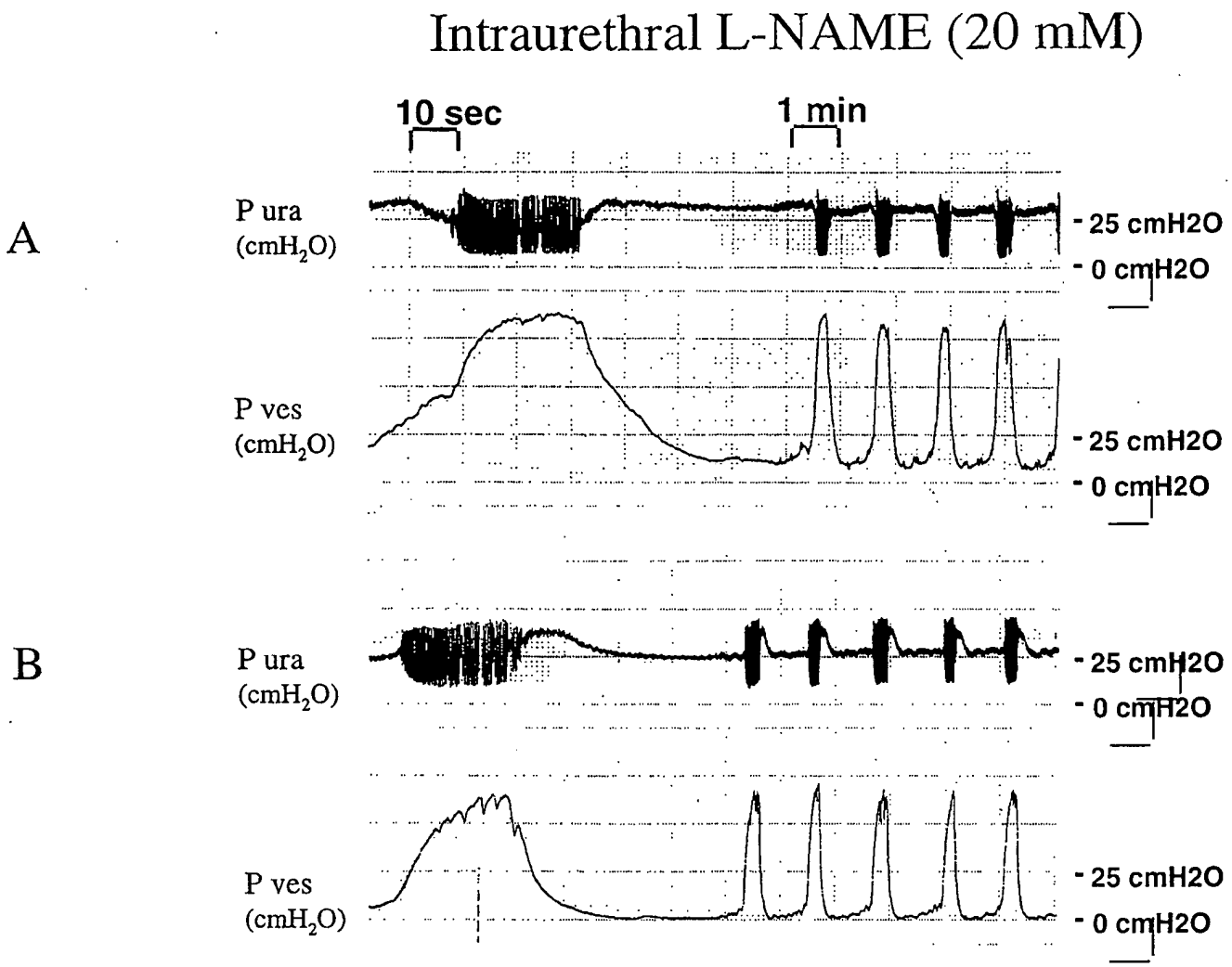

FIG. 5. Effects of intraurethral L-NAME (20 mM) on bladder pressure and urethral pressure in normal female rats. *A*, before treatment. *B*, after intraurethral administration of L-NAME. Bladder contraction frequency and urethral perfusion pressure were not significantly changed. L-NAME produced no change in nadir of urethral relaxation pressure.

the detrusor in the cat.[18] Mahony et al described two urethro-detrusor reflexes activated by urine flow across urethral mucosa that increased the excitability of the micturition reflex.[19] Hindmarsh proposed that urethral instability is often associated with unstable bladder contractions and bladder instability may be initiated from the bladder outlet region.[8] Thus in a woman with mixed stress and urge incontinence it would be appropriate to treat the stress incontinence as a strategy to improve or cure detrusor instability.

In cat experiments in which the bladder was surgically divided above the bladder neck, Kiruluta et al noted that urethral perfusion triggered spontaneous bladder contraction of such an intensity and frequency that bladder filling was not possible.[23] However, Sutherst and Brown did not detect such facilitatory reflexes in the human.[20] In this study, fluid was perfused via a double lumen catheter through urethral meatus. Thus the transurethral catheter may interfere with the activation of afferent receptors that respond to flow through the urethra.

Our experimental model provides many advantages to study the fascinating problem of urethral-bladder reflexes. Most previous studies isolated the bladder and urethra surgically, a method that could easily disrupt the bladder and urethra innervation. We have accomplished complete isolation between the bladder and urethra without surgery. Furthermore, our technique avoids any obstruction of the urethral outlet with transurethral catheters and has eliminated activation of the external urethral sphincter. By using this experimental model in which intravesical and urethral pressures were recorded simultaneously and independently, we have demonstrated that urethral afferents have an important modulatory role in the control of the micturition reflex.

Pharmacological activation of urethral afferents was produced by intraurethral administration of capsaicin. This stimulation produces a selective activation of primary afferent neurons.[24,25] Studies in the rat revealed that stimulation of capsaicin-sensitive primary afferents in the urethra leads to the activation/reinforcement of the activity of the external urethral sphincter.[26] Both functional[27] and immunohistochemical[28] data indicated that capsaicin sensitive primary afferents are present in the rat proximal urethra. In our in vivo study intraurethral capsaicin also altered bladder reflexes.

It has been suggested that the behavioral response induced by intravesical instillation of capsaicin is chiefly produced through the irritation of urethral afferents of pudendal origin rather than through pelvic bladder afferents.[29] In our study, intraurethral administration of 100 μM capsaicin produced a biphasic change in bladder activity. Initially after intraurethral capsaicin instillation the bladder contraction frequency

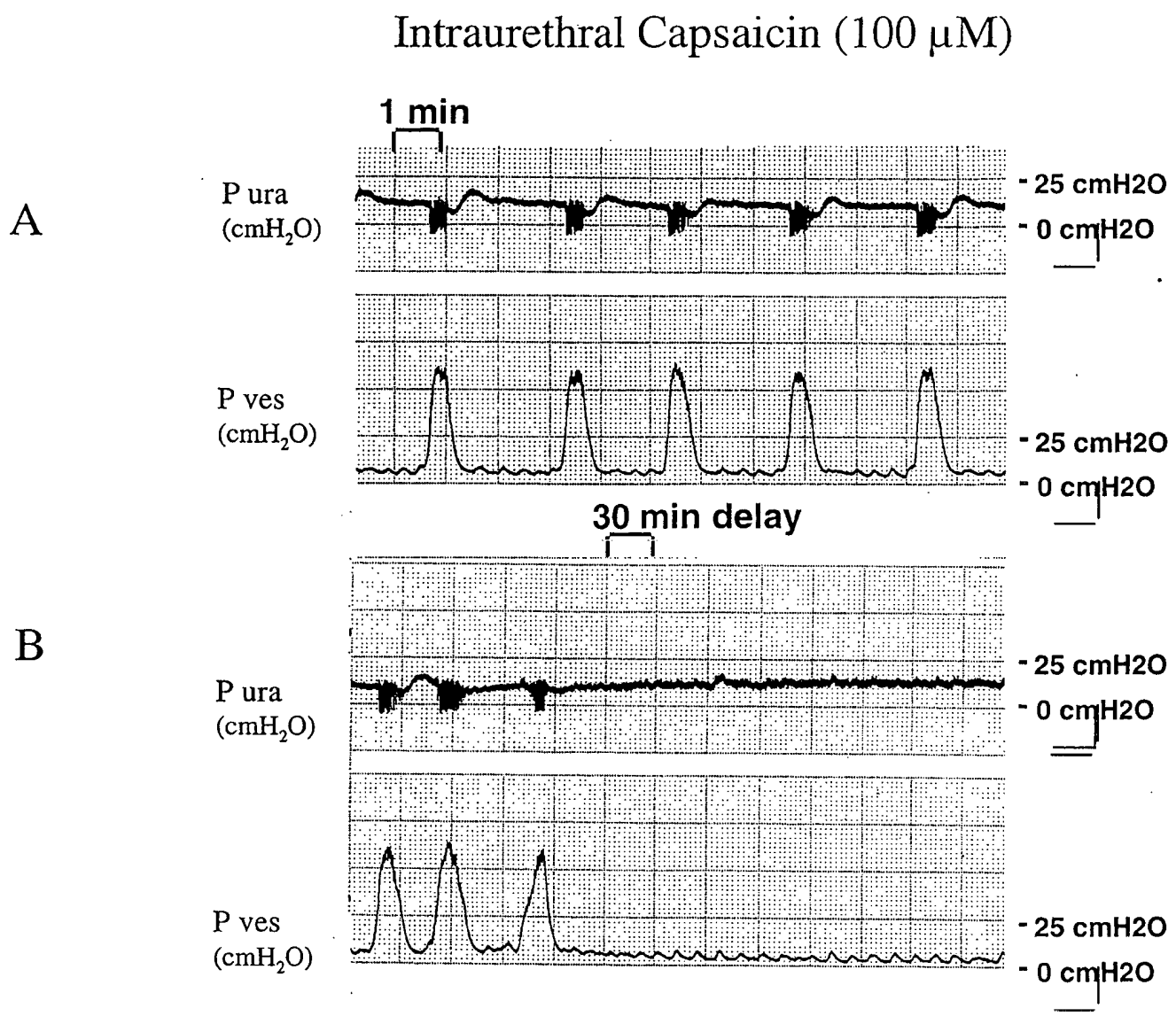

FIG. 6. Effects of intraurethral capsaicin on bladder pressure and urethral pressure in normal female rats. *A*, before treatment. *B*, after intraurethral administration of capsaicin (100 μM). Initially, intraurethral capsaicin instillation increased bladder contraction frequency but 30 minutes after continuous infusion activity was blocked.

increased but within 15 to 30 minutes the activity profoundly diminished. The results suggested that topical capsaicin stimulated and then desensitized the urethral afferent nerves. The latter effect decreased the excitability of the micturition presumably by eliminating urethral afferent input to the spinal cord.

We were able to demonstrate that fluid passing through the urethra could facilitate detrusor activity. Stopping the intraurethral saline infusion during control infusion caused a significant decrease in bladder contraction frequency. This finding suggests that mechanosensitive afferent nerves activated by fluid entering the urethra are able to increase the excitability of the micturition reflex.

Spontaneous rhythmic urethral activity occurring between reflex bladder contractions was observed during infusion at 0.075 ml. per minute, but not observed after stopping the infusion. It is possible that the contractions may be still present but cannot be seen because recording depends on some fluid in the urethra. The rhythmic urethral activity as reported by Persson and Andersson which cannot be inhibited by α-receptor blockade, atropine, or tetradotoxin might reflect a myogenic response to the slow constant saline infusion that was used to measure urethral activity.[30]

In a report by Low, bladder and urethral pressures were synchronously measured during bladder filling in 77 females with idiopathic instability. He found that 85% of patients developed urethral relaxation five seconds before detrusor contraction.[31] Low concluded that the involuntary detrusor contractions were preceded by a fall in urethral pressure, similar to that observed in normal voiding. Urethral instability was found in 15 patients with stress incontinence, in 23 patients with urge incontinence, and in 13 patients with combined stress and urge incontinence (that is, signs and symptoms of both stress and urge incontinence).[32] Thus, in women with urge incontinence and urethral instability, targeting treatment of the urethra would be a rational alternative.

We administered 1% lidocaine intraurethrally to block the urethral afferent nerve activity. Intraurethral lidocaine caused a significant decrease in the bladder contraction frequency without changing the amplitude of reflex bladder contractions. These results also strongly suggest that ure-

thral afferents are important in modulating the micturition reflex. Local anesthetics are believed to exert their effects primarily by blocking the entry of sodium into the axons.[33] Intraurethral lidocaine is unlikely to affect the efferent nerves since tissue penetration is poor and the efferent nerves are located deeper in the urethral wall.[34] Accordingly, intraurethral lidocaine most likely exerts its effects by blocking the submucosal afferent nerves.

Various evidence suggests that NO functions as neurotransmitter to relax the urethral smooth muscle.[35,36] Nitric oxide has been implicated in non-adrenergic, non-cholinergic urethral smooth muscle relaxation. The response to in vitro field stimulation is urethral relaxation that is blocked by NOS inhibitors[37] as well as urethral relaxation in vivo to parasympathetic nerve stimulation.[12]

The NO-synthesizing enzyme, nitric oxide synthase (NOS), is localized in nerve fibers of the detrusor, trigone, and urethra, but most prominently in the outflow region.[2] In this preliminary study we have shown that intraurethral administration of NO donors immediately decreased urethral perfusion pressure and maximum urethral pressure. This finding confirms previous reports suggesting that NO is likely to be one of the major neural inhibitory regulators of urethral tone in the rat and other species.[9] In addition, intraurethral administration of the NO donors also significantly decreased bladder contraction frequency. The mechanisms involved in this effect are not known, but one possibility is that NO can directly inhibit urethral afferent nerves because it is known to suppress the Ca^{+2} ion channel,[38] which may be important for activating urethral afferents.

Although the role of NO as a mediator of reflex urethral smooth muscle relaxation is evident, its effect on bladder activity is not well understood. Alterations in the L-arginine to NO pathway may be implicated in pathophysiologic mechanism leading to various voiding disorders. Recently, it was shown that inhibition of NO production causes bladder hyperactivity and decreases bladder capacity in the rat in vivo[39] and in the fetal lamb urinary bladder.[40] However, the origin of the detrusor muscle hyperactivity may be related to changes in urethra function.[8]

In this study, intraurethral administration of L-NAME did not have a significant effect on magnitude of reflex urethral relaxation and resting urethral tone. In other experiments, intravenous administration of NOS inhibitor reversibly decreased the magnitude and duration of reflex urethral relaxation.[10] A possible interpretation of these findings is that intraurethral L-NAME does not penetrate into the urethra in sufficient quantity to block neuronal NOS in the smooth muscle layers.

CONCLUSIONS

We found that passage of fluid through the urethra of the rat increases the frequency of reflex bladder contractions. Thus, in patients with stress urinary incontinence, leakage of urine into the proximal urethra could increase bladder activity by stimulating urethral afferents which in turn modulate the micturition reflex. This implies that stress incontinence can induce and/or increase detrusor instability. Nitric oxide, which has an important role in the reflex urethral smooth muscle relaxation during micturition, may also have a role in urethral afferent function. These findings have significant implications for the treatment of patients with mixed incontinence. Correction of stress incontinence by surgery or pelvic floor exercise in patients with mixed incontinence may resolve the detrusor instability.

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PERSISTENCE AND SURVIVAL OF AUTOLOGOUS MUSCLE DERIVED CELLS VERSUS BOVINE COLLAGEN AS POTENTIAL TREATMENT OF STRESS URINARY INCONTINENCE

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ABSTRACT

Purpose: We explored the use of autologous muscle derived cells as a method of treating stress urinary incontinence. We determined whether urethral muscle derived cell injection is feasible and compared it with bovine collagen injection.

Materials and Methods: Muscle derived cells isolated from female Sprague-Dawley rats were first transduced with retrovirus carrying the transgene for β-galactosidase. We injected approximately 1 to 1.5 × 10^6 cells into the bladder wall and proximal urethra of 6 autologous animals. Tissue was harvested after 3 and 30 days, sectioned, stained for fast myosin heavy chain and assayed for β-galactosidase. To compare muscle derived cell and bovine collagen injections 100 μl. of commercially available bovine collagen were also injected in Sprague-Dawley female rats. Tissue was harvested in 3 animals each after 3 and 30 days, sectioned and stained for trichrome. Subsequently, 3 adult SCID mice were used to compare the level of transgene expression at each time point after injecting 1.5 × 10^6 cells per injection, which were transduced with adenovirus carrying the transgene for β-galactosidase.

Results: A large number of cells expressing β-galactosidase were observed in the bladder and urethral wall 3 and 30 days after autologous cell injection in Sprague-Dawley rats. The persistence of primary muscle derived cells at 3 days was similar to that of collagen. However, at 30 days there was significant cell persistence while only a minimal amount of injected bovine collagen was detectable. Approximately 88% of the β-galactosidase expression at day 3 remained at day 30 in SCID mice.

Conclusions: We present 2 new findings important for the emerging field of urological tissue engineering, including the feasibility of injecting autologous skeletal muscle derived cells into the lower urinary tract and the greater persistence of such injected cells versus injected bovine collagen. Therefore, autologous muscle derived cell injection may be an attractive alternative treatment option for stress urinary incontinence.

KEY WORDS: urethra; collagen; urinary incontinence, stress; stem cells; transplantation, autologous

Adult bladders and urethras lack stem cells and cannot regenerate effectively.[1] The lack of bladder and urethral smooth muscle regenerative ability may contribute to lower urinary tract dysfunction. For example, stress urinary incontinence may develop as a result of intrinsic sphincter muscle deficiency. Impaired contractility of smooth muscle occurs in aging, neuropathic bladder and benign prostatic hyperplasia. There is no effective pharmacological intervention for either condition.[2,3]

In contrast to smooth muscle, skeletal muscle is constantly undergoing repair because of undifferentiated myogenic cells, known as satellite cells.[4] These cells are fusion competent skeletal muscle precursors. When differentiated, they fuse to form myofibers capable of muscle contraction. We and others previously demonstrated the ability to harvest muscle derived cells containing satellite cells and myoblasts from skeletal muscle biopsy.[5,6] To date the only clinical application of muscle-based cellular transplants involves muscle derived cells transplantation for Duchenne muscular dystrophy.[7] In basic research muscle derived cells have been used for treating myocardial infarction models and for delivering secretory nonmuscle protein products, such as human growth hormone and coagulation factor IX, to the circulation.[8,9] In addition, when muscle derived cells differentiate and form myofibers, they become postmitotic, resulting in long-term persistence.[10] Thus, cells derived from skeletal muscle may be used to treat stress urinary incontinence and/or impaired bladder contractility.

The field of urological tissue engineering has recently achieved several important milestones with the development of a tissue engineered bladder replacement and an injectable agent for vesicoureteral reflux.[11–13] In this study we used autologous injections of muscle cells harvested from subject skeletal muscle, which may proliferate and functionally improve the bladder and sphincter. We call this procedure cellular uromyoplasty. We have reported preliminary data demonstrating that cultured mouse muscle derived cells injected into the bladder and urethral wall in rats were histologically identifiable shortly after injection.[14,15] However, to our knowledge long-term persistence of muscle derived cells placed by autologous injection in the lower urinary tract has not been studied to date. Therefore, we performed experiments to explore the feasibility of transplanting autologous muscle derived cells into the bladder and urethral wall, and examine the survivability of transplanted cells. We also com-

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pared primary muscle derived cell transplantation with bovine collagen injection.

MATERIALS AND METHODS

Experiments were performed in 6 to 8-week-old female Sprague-Dawley rats and adult SCID mice in accordance with National Institutes of Health requirements and recommendations.[16] This project was approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh and the University of Pittsburgh (protocol 14–98). Only certified viral-free animals were used.

In 6 Sprague-Dawley rats muscle derived cells obtained from the gastrocnemius muscle were injected into the bladder or urethra in the same host animals to evaluate the feasibility of autologous muscle derived cell injection. This group was compared with 6 other rats that received collagen injection into the bladder and urethra. In addition, we performed an experiment using 6 SCID mice to evaluate quantitatively the level of adenovirus mediated β-galactosidase expression in muscle derived cells injected into the bladder wall. We used SCID mice only in this experiment because using immunodeficient animals would circumvent the possible rejection of adenoviral vectors encoding marker gene transduced in muscle derived cells, which may cause a discrepancy between β-galactosidase expression and the number of surviving muscle derived cells in immunocompetent animals. SCID mice received injections of cells obtained from normal neonatal mice.

Purification of muscle derived cells. A muscle biopsy specimen was obtained from the gastrocnemius muscle of adult female Sprague-Dawley rats and from the whole hind limb muscle of normal neonatal mice under sterile conditions. Biopsied muscle was minced into a coarse slurry using razor blades. Cells were enzymatically dissociated by adding 0.2% collagenase type XI for 1 hour at 37C, 240 units of grade II dispase for 45 minutes and 0.1% trypsin for 30 minutes. Dissociated cells were preplated on collagen coated flasks and the muscle population in each flask was evaluated by desmin staining. Based on previous experiments at our laboratory the first preplated flasks contain a majority of fibroblasts and the last flasks are highly enriched with muscle derived cells.[5] The highest enriched preplate was used in our experiment. The proliferation medium was Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, 10% horse serum, 0.5% chick embryo extract and 1% penicillin-streptomycin. The fusion medium was DMEM supplemented with 2% fetal bovine serum and 1% antibiotic solution (penicillin-streptomycin).

Muscle derived cell preparation. For virus transfection muscle derived cells were plated at a density of approximately 1 to 1.5 × 10^6 in T 75 flasks, rinsed in Hank's balanced salt solution (HBSS) and incubated with the retroviral vector (1 × 10^7 to 1 × 10^9 colony-forming units per ml.) or adenoviral vector (3 × 10^{10} colony-forming units per ml.) before suspension in 5 ml. of DMEM for 4 hours at 37C. Infection with the retrovirus was done in the presence of 8 μg./ml. polybrene to improve attachment to the culture cells. After this initial incubation period 10 ml. of proliferation medium were added for an additional 24 hours of incubation at 37C. At 24 hours after infection the viral suspensions were removed and cells were rinsed with HBSS. Muscle derived cells were detached using 0.25% trypsin for 1 minute and centrifuged for 5 minutes at 3,500 rpm. Cell pellets was reconstituted in 20 μl. of HBSS.

Retroviral vectors. The retroviral vector used was MFG-NB.[17] This vector contains the modified LacZ gene nls-LacZ, which includes a nuclear localization sequence cloned from the simian virus 40 large tumor antigen. It is transcribed from the long terminal repeat. The titer of the viral stock was 1 × 10^7 to 1 × 10^9 colony-forming units per ml. Viral stock was grown in the laboratory.[18] This retrovirus was used in the Sprague-Dawley rat autologous cell injection experiments.

Adenoviral vectors. The E1-E3 deleted recombinant adenovirus had the β-galactosidase gene under the control of the human cytomegalovirus promoter, followed by the simian virus 40 t-intron and polyadenylation signal. This adenovirus was used to measure the level of transgene expression in SCID mice.

Muscle derived cells and collagen injection in animals. Subjects were housed in an approved viral gene therapy facility at Children's Hospital of Pittsburgh. After surgical preparation a low midline incision was made to expose the bladder and urethra. A total of 20 μl. of muscle derived cells suspended in HBSS solution (approximately 1 to 1.5 × 10^6 cells per 20 μl.) or 100 μl. of commercially available bovine collagen were injected into the proximal urethra or lateral bladder wall of Sprague-Dawley rats with a 50 μl. Hamilton microsyringe for cell injection in 6 subjects and a 500 μl. disposable syringe with a 28 gauge needle for collagen injection in 6. For comparing primary cell and collagen injections we evaluated 3 rats per group at each time point of 3 and 30 days. Three adult SCID mice were then used to compare the level of transgene expression at each time point of 3 and 30 days after injecting 1.5 × 10^6 cells per injection, which were transduced with adenovirus carrying the LacZ reporter gene. Muscle derived cells used for SCID mice were obtained from normal neonatal mice.

Tissue harvest and histology. Animals were sacrificed 3 and 30 days after cell or collagen injection, and the bladder and urethra were removed. Tissue was then snap frozen using 2-methylbutane pre-cooled in liquid nitrogen. Analysis of the sections included hematoxylin and eosin, and trichrome staining as well as X-gal staining for the β-galactosidase reporter gene to determine the location of the gene transfer area and the viability of injected cells. The area around each injection site was stained, examined microscopically and photographed.

LacZ staining by histochemical technique. Cryostat sections of the injected tissue were stained for LacZ expression. They were first fixed with 1% glutaraldehyde for 1 minute and rinsed twice in phosphate buffered saline (PBS). They were then incubated in X-gal substrate (0.4 mg./ml. 5-bromo-chloro-3-indolyl-β-D-galactoside, 1 mM. magnesium chloride and 5 mM. $K_4Fe(CN)_6$-5 mM. $K_3Fe(CN)_6$ in PBS overnight at 37C.

Immunohistochemical staining for fast myosin heavy chain. A monoclonal antibody specific for fast myosin heavy chain was used in this study. Tissues were fixed with cold acetone at −20C for 2 minutes and blocked with 5% horse serum for 1 hour. Sections were incubated overnight at room temperature in a humid chamber with primary antibodies (1/400 monoclonal mouse antiskeletal myosin, fast, M.-4276) in PBS, pH 7.4. After 3 rinses in PBS sections were incubated with an antimouse secondary antibody conjugated to Cy3 immunofluorescence (1/200).

Immunohistochemical staining for desmin. A monoclonal antibody specific for desmin was used. Cultured cells were fixed with cold methanol at −20C for 1 minute and blocked with 5% horse serum for 1 hour. Cells were incubated overnight at room temperature in a humid chamber with primary antibodies (1/200 monoclonal mouse antidesmin) in PBS, pH 7.4. After 3 rinses in PBS cells were incubated with an antimouse secondary antibody conjugated to Cy3 immunofluorescence (1/200).

Cell culture. Primary myoblasts were plated in 35 mm. collagen coated dishes in the proliferating medium. After 24 hours the proliferating medium was replaced by fusion medium. Cells were maintained in fusion medium with a daily medium change until myoblasts differentiated into myotubes.

O-nitrophenyl-β-D-galactopyranoside (ONPG) assays for

β-galactosidase activities. This technique was used to achieve better quantitation and comparison of the transgene expression level in injected bladders.[19] Injected bladders were frozen in pre-cooled isopentane and homogenized in 0.25 M. tris hydrochloride, pH 7.8. Homogenates were then disrupted by a series of 3 freeze-thaw cycles. After the last thaw the homogenates were centrifuged at 14,000 × gravity for 5 minutes at 4C. The supernatant containing the cell extract was removed and transferred to a clean Eppendorf tube, and 30 μl. were mixed with 66 μl. of 4 mg./ml. ONPG dissolved in 0.1 M. sodium phosphate, pH 7.5, 3 μl. of 4.5 Mβ-mercaptoethanol dissolved in 0.1 M. magnesium chloride and 201 μl. 0.1 M. sodium phosphate. The mixture was incubated at 37C for 30 minutes and the reaction was stopped by adding 500 μl. of 1 M. sodium carbonate. Optical density was read on a spectrophotometer at a wavelength of 420 nm. The amount of β-galactosidase activity in the injected tissue (ng. of β-galactosidase per whole bladder) was then calculated by referring to a standard calibration curve.

Statistical analysis. Values are reported as the mean plus or minus standard deviation. The Student t test was done to compare groups with $p < 0.05$ considered statistically significant.

RESULTS

Adult skeletal muscle is composed of intact myofibers surrounded by quiescent immature reserve satellite cells.[4] In addition, we recently identified muscle derived cells containing satellite cells biopsied from mouse hind limb skeletal muscle.[20] Immunohistochemical study for desmin showed cells derived from skeletal muscle obtained by biopsy at 80% to 90% confluence after approximately 6 days in vitro (fig. 1). After reaching approximately 70% confluence muscle derived cells began to differentiate chemically and become committed to fuse and form mature multinucleated skeletal myotubes, similar to those in intact muscle. These elongated myotubes then coalesced in vivo to form myofibers.

At 3 and 30 days after autologous muscle derived cells transplantation in rats the bladder and urethra were harvested, examined grossly and subjected to histological analysis. We observed a large number of cells expressing β-galactosidase in the bladder and urethral walls at each time point after autologous muscle derived cell injection in rats. Moreover, injected muscle derived cells comprised a mass that protruded toward the lumen of the bladder and urethra.

FIG. 1. Photomicrograph of cultured muscle derived cells shows immunohistochemical staining for desmin 6 days after isolation from skeletal muscle of female Sprague-Dawley rat. Multinucleated myotubes resulting from cell fusion are apparent. Reduced from ×200.

Histological examination revealed no apparent sign of inflammation or tissue damage, such as the infiltration of inflammatory cells (platelets, macrophages and monocytes) at the injection site (fig. 2). Fluorescence microscopy of tissue sections from the bladder and urethra injected with autologous cells revealed myotubes that stained positive for fast myosin heavy chain, while no positive staining was observed outside of the injection area (fig. 3).

When comparing muscle derived cell versus collagen injection in rats, the persistence of the injected substances was similar at 3 days. However, at 30 days there was significant persistence of cells that resulted in a bulging mass in the urethral lumen, while only scant bovine collagen was detectable in collagen injected animals (fig. 4). We also quantified the size of nodules created by cell or collagen injection. Average diameter plus or minus standard deviation of the nodules due to cell injection 3 and 30 days after injection was 1.03 ± 0.05 and 0.84 ± 0.08 mm., respectively. The mean diameter of nodules caused by collagen injection was 0.78 ± 0.07 mm. 3 days after injection, which decreased to 0.15 ± 0.05 mm. at 30 days. Nodule size due to cell and collagen injection did not differ 3 days after injection. However, at 30 days nodules caused by cell injection were significantly larger than those caused by collagen injection ($p < 0.05$).

Furthermore, we performed the ONPG assay to assess quantitatively the level of β-galactosidase expression in transduced muscle derived cells injected into the bladder wall. These experiments involved SCID mice because we measured adenovirus mediated β-galactosidase expression to evaluate the level of survival of muscle derived cells in immunodeficient animals. After cell injection the level of β-galactosidase expression was well maintained. Only a 12% decrease was detected at 30 days compared with the level at 3 days (fig. 5). In addition, this difference in β-galactosidase expression was not statistically significant.

DISCUSSION

The goal of these experiments was to establish the basic methodology for future clinical applications of muscle derived cell tissue engineering for treating urological dysfunction. We successfully isolated muscle derived cells from adult animals, and injected cultured cells into the urethral and bladder walls, resulting in the formation of myotubes in the smooth muscle layers. We noted approximately 88% survival of muscle derived cells injected into the bladder wall, as measured by cell mediated reporter gene expression at 1 month. The transplantation of autologous muscle derived cells in immunocompetent adult rats allowed us to demonstrate the feasibility of this treatment without the usual immunological problems of nonautologous transplantation.

Bovine collagen manufactured in the form of an injectable paste is the most common and most successful material used for correcting stress incontinence. Collagen gained Food and Drug Administration approval in 1993 for treating intrinsic sphincter deficiency. Approximately 70% of patients are improved with collagen injection, of whom up to 30% are cured or significantly improved and 40% are only partially improved, while the remaining 30% are not helped by collagen injection.[21] However, there are some major disadvantages to collagen injection. Collagen is often reabsorbed, which adversely affects a successful outcome and requires repeat injections in the majority of patients. An average of 3 collagen injections is needed to achieve partial or complete improvement. Also, 5% of patients are allergic to bovine collagen and in most who are injected with collagen antibodies develop to bovine antigens.[22]

In contrast to collagen, muscle derived cells demonstrated improved persistence in our study, as indicated by quantitative analyses of a mass effect after cell and collagen injection. These results support the feasibility of muscle derived cell

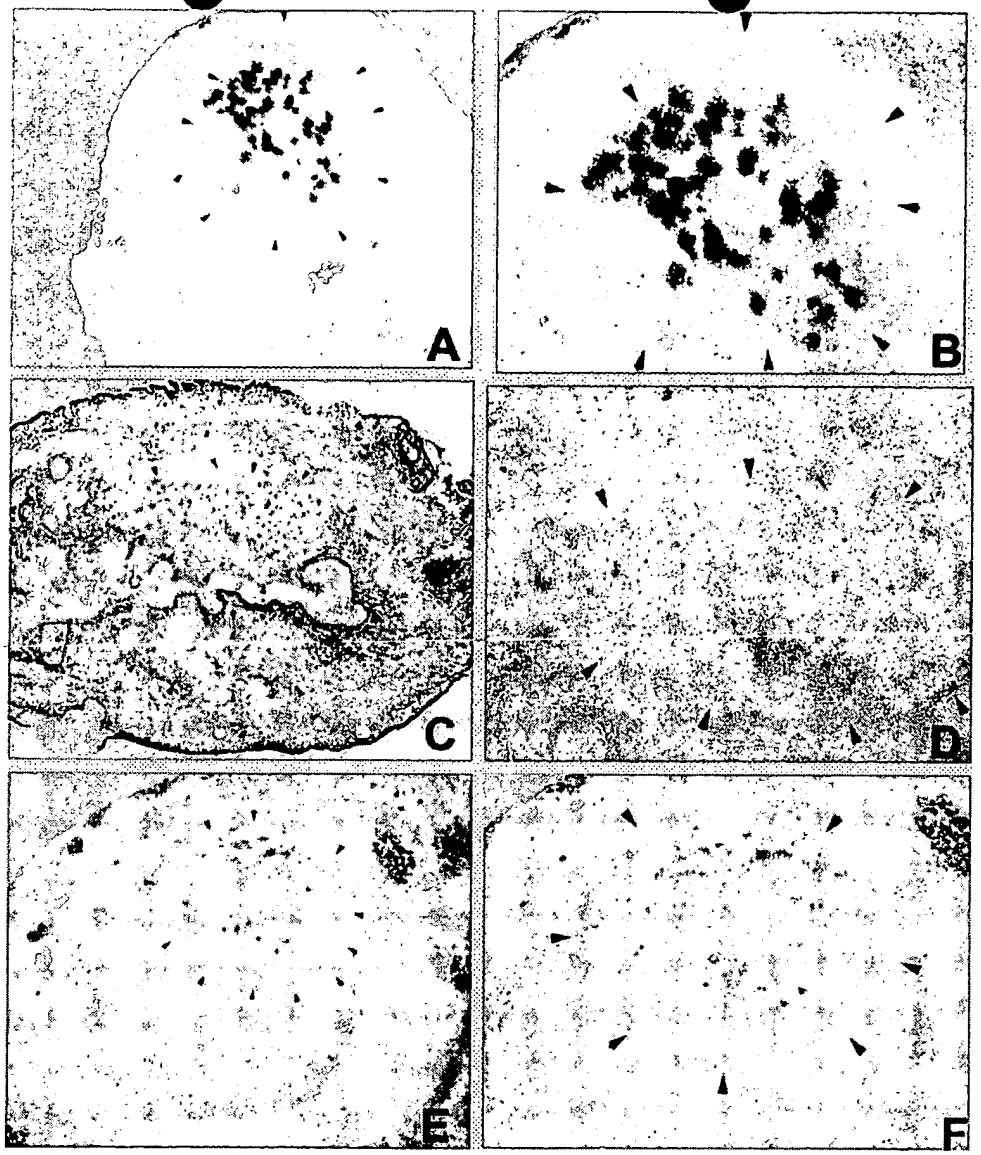

FIG. 2. Photomicrographs of bladder and urethral sections demonstrate large number of LacZ positive muscle derived cells in Sprague-Dawley rats 3 and 30 days after injection. *A* and *B*, day 3 after injection of muscle derived cells into bladder wall. *C* and *D*, day 30 after injection of cells into bladder wall. *E* and *F*, day 30 after injection of cells into urethral wall. *A*, *C* and *E*, reduced from ×40. *B*, *D* and *F*, reduced from ×100.

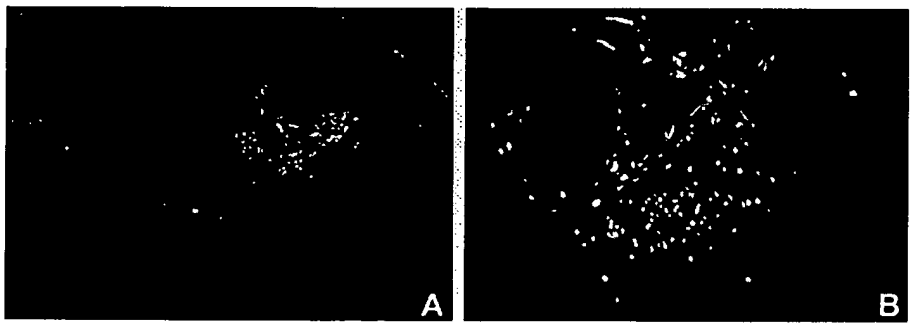

FIG. 3. Photomicrographs of bladder sections show many myotubes positively staining for fast myosin heavy chain 30 days after muscle derived cell injection into bladder wall of Sprague-Dawley rat. *A*, reduced from ×40. *B*, reduced from ×100.

injection as a possible treatment for stress urinary incontinence. The injection of the muscle derived cells in the bladder and urethral walls did not lead to any adverse effects. Thus, we hypothesize that autologous muscle derived cells may be a highly desirable injectable substance for treating stress incontinence because they are nonallergenic and have long-term persistence. It is also possible that autologous muscle derived cell injection will decrease the cost of currently available injectable materials, including collagen, since cells may be obtained from the host patient.

Although to our knowledge we report the first injections of skeletal muscle derived cells into the urinary tract, there

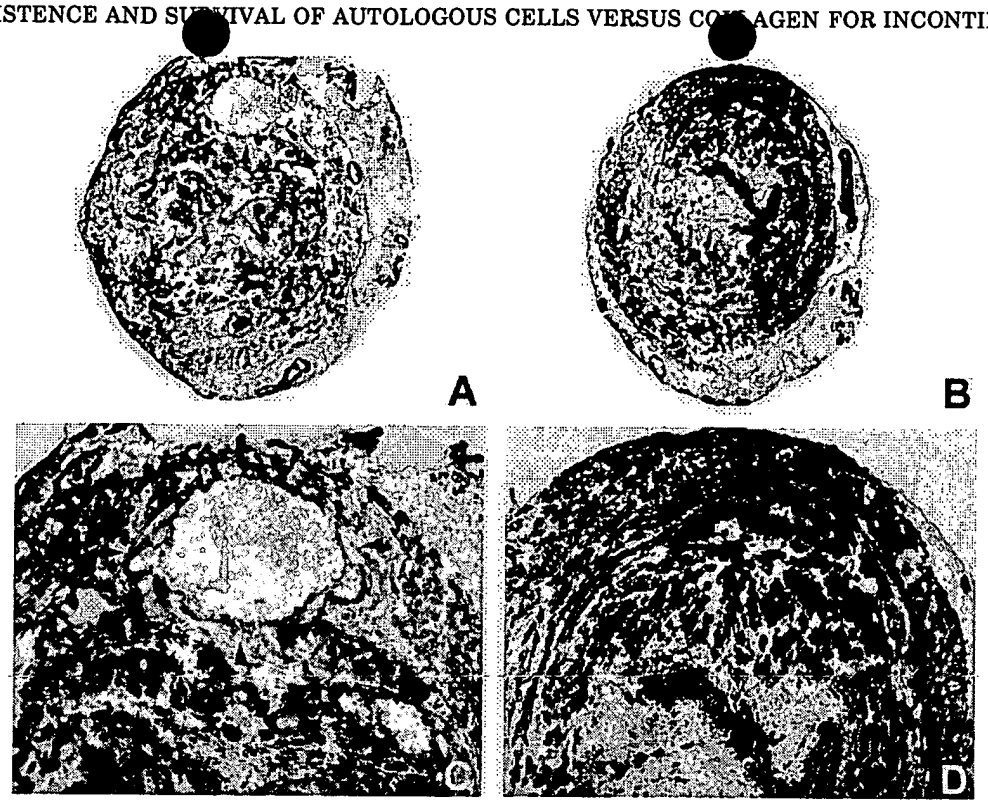

FIG. 4. Photomicrographs of urethral sections reveal collagen deposits after injection of collagen into urethral wall in Sprague-Dawley rats. *A* and *C*, day 3 after injection. *B* and *D*, by 30 days after injection almost all injected collagen was absorbed. Masson trichrome stain. *A* and *B*, reduced from ×40. *C* and *D*, reduced from ×100.

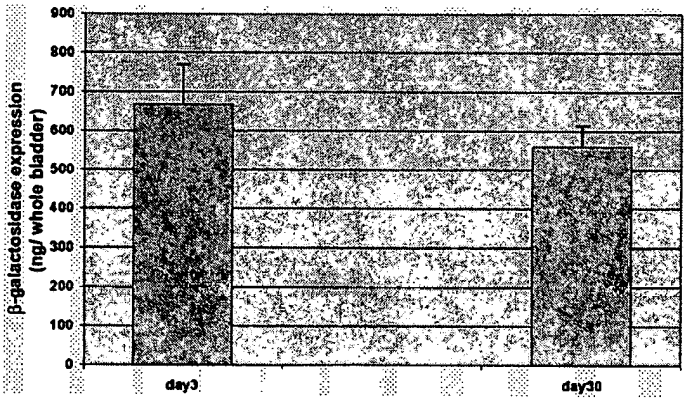

FIG. 5. Quantitative analysis of adenovirus mediated β-galactosidase expression in muscle derived cells injected into bladder and urethra of SCID mice. β-Galactosidase activity was evaluated by LacZ expression in ONPG assay. Of transgene expression observed at 3 days 88% remained 30 days after injection.

have been reports of gene therapy and skeletal myoblast therapy for promoting myocardial regeneration.[23–26] Muscle derived cells differentiate into myofibers and then myotubes. Since myotubes are postmitotic, cells do not continue to grow or spread. Therefore, it seems unlikely that muscle derived cells would grow uncontrollably into the lower urinary tract or adjacent structures and, thereby, cause urethral outlet obstruction. This hypothesis is consistent with the results of our experiments. In addition to the ability of muscle derived cells to differentiate into myotubes, they may also improve the function of injected organs because they contain satellite cells with stem cell properties that allow differentiation into various cell types (possibly smooth muscle cells) based on the host environment,[27] may improve diverse tissue function[28] and promote innervation.[29] For example, the intramuscular injection of muscle derived cells engineered to secrete bone morphogenetic protein has induced ectopic bone formation.[30] More importantly, we have observed that muscle derived cells were in fact actively participating in bone formation in vivo[31] and were capable of differentiating into cartilage and bone for orthopedic repair. This latter result shows that the ability of muscle derived cells to differentiate into other lineages may represent an important feature for the clinical use of these cells. In addition, since muscle derived cells are capable of delivering new genes, they may be engineered to secrete many trophic substances to improve muscle regeneration and enhance muscle strength, leading to improved muscle healing after injury.[32] Therefore, it is possible to assume that autologous injection of muscle derived cells in the lower urinary tract may not only have a mass effect for increasing mechanical resistance in the urethra, but also improve functional properties of the bladder and urethra if these cells acquire the properties of smooth muscle fibers and/or enhance innervation of injected target organs. In future studies we plan to investigate whether muscle derived cell transplantation improves the muscle structure and physiological function of compromised bladder smooth muscle and sphincter muscle.

CONCLUSIONS

We have demonstrated 2 new results in urological tissue engineering, including the feasibility of injecting autologous skeletal muscle derived cells into the lower urinary tract and the greater persistence of injected muscle derived cells versus bovine collagen. Autologous muscle derived cellular uromyoplasty may be an attractive treatment alternative for stress urinary incontinence.

Viral stock was grown in the laboratory of Dr. Johnny Huard, E1-E3 deleted recombinant adenovirus was provided by Dr. I. Kovesdi, and Ryan Pruchnic and Marcelle Pellerin provided technical assistance.

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ELSEVIER

MUSCLE-DERIVED CELL TRANSPLANTATION AND DIFFERENTIATION INTO LOWER URINARY TRACT SMOOTH MUSCLE

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ABSTRACT

Objectives. To explore the feasibility of primary skeletal muscle-derived cell (MDC)-based tissue engineering and gene transfer into the lower urinary tract and to explore whether the injected primary skeletal MDCs can persist and differentiate into myotubes and myofibers in the bladder wall.

Methods. Primary MDCs isolated from normal mice were first transduced with adenovirus encoding the expression of the beta-galactosidase reporter gene. Adult severe combined immunodeficiency mice (n = 12) were used in this study. The MDCs were injected into the right and left lateral bladder walls with a 10-μL Hamilton microsyringe. The amount of injected MDCs ranged from 1 to 1.5 $\times$ 10^6 cells. The tissue was harvested after 5, 35, and 70 days, sectioned, stained for fast myosin heavy chain, and assayed for beta-galactosidase expression.

Results. We observed a large number of cells expressing beta-galactosidase in the bladder wall at each time point. Many myotubes and myofibers expressing beta-galactosidase and positively stained for fast myosin heavy chain were also seen in the bladder wall at 35 and 70 days after injection. Additionally, the size of the injected MDCs significantly increased during the course of the study ($P < 0.05$).

Conclusions. We have demonstrated the long-term survival and beta-galactosidase expression of MDCs injected into the bladder wall. Moreover, our results suggest that some injected MDCs can differentiate into myofibers. These results suggest that MDCs can be a desirable substance for tissue engineering and an ex vivo method for gene transfer into the lower urinary tract. UROLOGY **57**: 826–831, 2001.

Bladder dysfunction is a devastating medical problem. Presently, clinicians are extremely limited in their ability to treat impaired detrusor contractility. No effective medications to improve detrusor contractility are available.[1] We are exploring a new treatment for bladder dysfunction using skeletal muscle-derived cells (MDCs). The design of this experiment was to provide proof of the concept for future clinical applications of MDC-based tissue engineering and gene therapy for urologic dysfunction.

This study was supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases (NIH RO1 DK55387) and the Pittsburgh Tissue Engineering Initiative.

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The delivery of growth factors, cells, and therapeutic genes promises to revolutionize a medical field historically limited to biomechanical approaches. Although gene therapy approaches were designed for correcting genetic disorders, this technology may be amenable to treating acquired diseases and improving the healing of various conditions of the lower urinary tract. Advances in cellular and molecular biology have identified a number of proteins, such as insulin-like growth factor and nerve growth factor, that may potentially improve detrusor contractility.[2,3] Gene transfer offers a solution to the problems associated with delivering these proteins to the lower urinary tract.

Muscle-based gene complementation has predominantly been aimed at diseases, such as Duchenne muscular dystrophy,[4,5] and gene transfer for non-muscle applications, such as hemophilia and growth hormone deficiency.[6,7] The theory behind muscle-based tissue engineering is based on the

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unique biology of skeletal MDCs. Skeletal muscle contains satellite cells that are resting mononucleated precursor cells. Satellite cells are able to fuse and form post-mitotic, multinucleated myotubes and myofibers capable of persistent gene expression.[8] Moreover, skeletal muscle may contain a population of mesenchymal stem cells, which are capable of differentiating into several different lineages.[9] Consequently, MDCs may also be capable of regenerating many different tissues. Tissue engineering based on these cells not only facilitates gene delivery but may also supply the stem cells needed for healing.

In the urologic field, Atala and coworkers[10,11] have demonstrated successful tissue engineering and gene delivery using an endothelial tissue, as well as the development of tissue engineered bladder replacement and an injectable agent.[12–14] Tissue engineering holds the promise of delivering new solutions, as well as gene therapy, to the urologic field.

The present experiment was performed to explore the feasibility of primary MDC-based tissue engineering and gene transfer into the lower urinary tract.

MATERIAL AND METHODS

Animals and MDC Injection

All experiments were performed on mature severe combined immunodeficiency (SCID) mice in accordance with the requirements and recommendations in the Guide for the Care and the Use of Laboratory Animals (U.S. Public Health Service, National Institutes of Health Publication No. 85-23, 1985). This project was approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and the University of Pittsburgh (Protocol Nos. 14-98). Only certified viral-free animals were used. The animals were housed in an approved viral gene therapy facility (Children's Hospital of Pittsburgh). After surgical preparation, a low midline incision was made to expose the bladder and urethra. Twenty microliters of MDCs suspended in Hank's balanced salt solution (1 to 1.5×10^6 cells per 20 μL) was injected into the right and left lateral bladder walls with a 10-μL Hamilton microsyringe.

Purification of Primary MDCs

The forelimbs and hindlimbs were removed from neonatal normal mice, and the bone was dissected. The remaining muscle mass was minced into a coarse slurry using razor blades. Cells were enzymatically dissociated by the addition of collagenase-type XI 0.2% for 1 hour at 37°C, dispase (grade II 240 units) for 45 minutes, and trypsin 0.1% for 30 minutes. The muscle cell extract was preplated on collagen-coated flasks, and the myogenic population in each flask was evaluated by desmin staining, a myogenic marker. On the basis of previous experiments, the first preplate (PP1) flasks contained mostly fibroblasts. PP1 represented a population of MDCs that adhered in the first hour after isolation, PP2 in the next 2 hours, PP3 in the next 18 hours, and subsequent preplates were obtained at 24-hour intervals (PP4 to PP6). PP6 was highly enriched with desmin-expressing MDCs and displayed a better ability to fuse with host myofibers.[15] PP6 was used in this experiment. The proliferation medium was DMEM with 10% fetal bovine serum, 10% horse serum, 0.5% chick embryo extract, and 1% penicillin/streptomycin; the fusion medium was DMEM supplemented with 2% fetal bovine serum and 1% penicillin/streptomycin. All culture medium supplies were purchased through Gibco Laboratories (Grand Island, NY).

MDC Preparation and Transduction with Adenovirus

Sufficient adenovirus stock was supplemented into each well containing MDCs to achieve a multiplicity of infection equal to 50 (MOI = 50). Cells were incubated with the adenovirus carrying the expression of lacZ reporter gene for 3 hours at 37°C to allow infection. Proliferating media were then added to each well. Plates were placed at 37°C overnight. The MDCs transfected with adenovirus carrying the lacZ reporter gene showed approximately 100% positive lacZ expression in vitro. At 24 hours after infection, the MDCs were detached using trypsin (0.25%) for approximately 1 minute and centrifuged for 5 minutes at 3500 rpm, and the MDC pellet was reconstituted with 20 μL Hank's balanced salt solution (Gibco-BRL). Cells were injected into SCID mice (n = 12) at a range of 1 to 1.5×10^6 cells per 20 μL. The injected tissue was harvested on days 5, 35, and 70 after injection and flash frozen in liquid nitrogen. Four mice were killed at each time point, and the tissue was sectioned in its entirety at 10 μm thickness using a cryostat, histochemically stained for beta-galactosidase, and then counterstained with hematoxylin-eosin.

Adenoviral Vectors

The adenovirus, an E1-E3 deleted recombinant adenovirus obtained through Dr. I. Kovesdi (Gene Vec, Rockville, Md), contained the beta-galactosidase gene under the control of the human cytomegalovirus promoter followed by the SV40 t-intron and polyadenylation signal.

Tissue Harvest and Histologic Examination

The animals were killed at 5, 35, and 70 days after myoblast injection and the bladders removed. The tissues were snap frozen using 2-methylbutane precooled in liquid nitrogen. The analysis of the sections included counterstaining with hematoxylin-eosin staining, X-gal staining for the beta-galactosidase reporter gene to determine the location of the gene transfer area, and the viability of the injected MDCs. Some sections were analyzed for fast myosin heavy chain by immunohistochemistry. The area around each injection site was stained, examined microscopically, and photographed.

lacZ Staining by Histochemical Technique

The cryostat sections of the injected tissue were stained for lacZ expression. First, they were fixed with 1.0% glutaraldehyde (Sigma Chemical, St. Louis, Mo) for 1 minute and rinsed twice in phosphate-buffered saline (PBS). Finally, they were incubated in X-gal substrate [0.4 mg/mL 5-bromo-chloro-3-indolyl-beta-D-galactoside] (Boehringer-Mannheim, Indianapolis, Ind), 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$ in PBS overnight (37°C).

Immunohistochemical Staining for Fast Myosin Heavy Chain

A monoclonal antibody specific for fast myosin heavy chain was used in this study. The tissues were fixed with cold acetone (−20°C) for 2 minutes and blocked with 5% horse serum for 1 hour. The sections were incubated overnight at room temperature in a humid chamber with primary antibodies (1/400 monoclonal mouse antiskeletal myosin [fast]; M-4276; Sigma Chemical) in PBS, pH 7.4. After three rinses in PBS, the sections were incubated with an anti-mouse secondary antibody

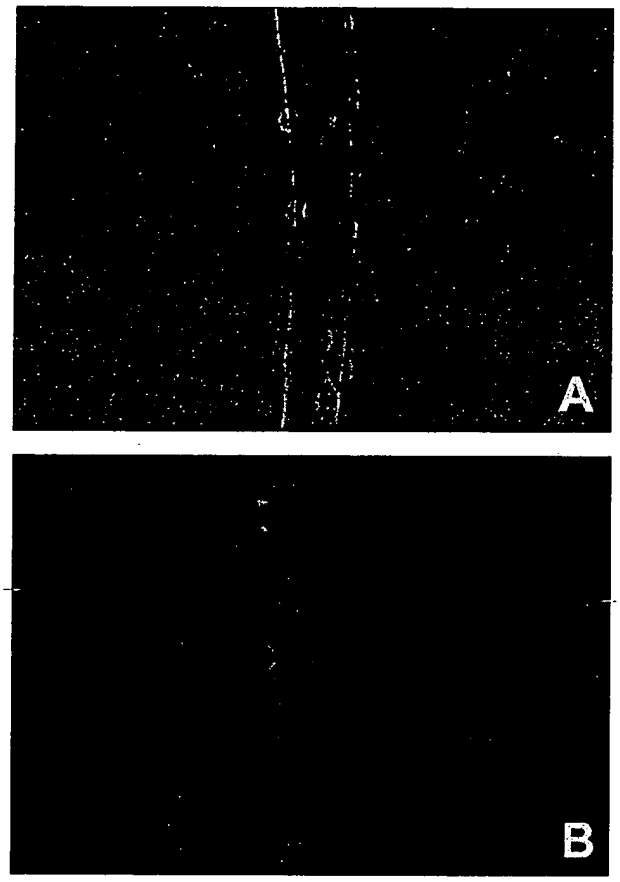

FIGURE 1. *(A) Single muscle fiber prepared from normal mice culture in vitro. (B) M-cadherin staining showed satellite cells located on the periphery of the muscle fiber. Original magnification ×40.*

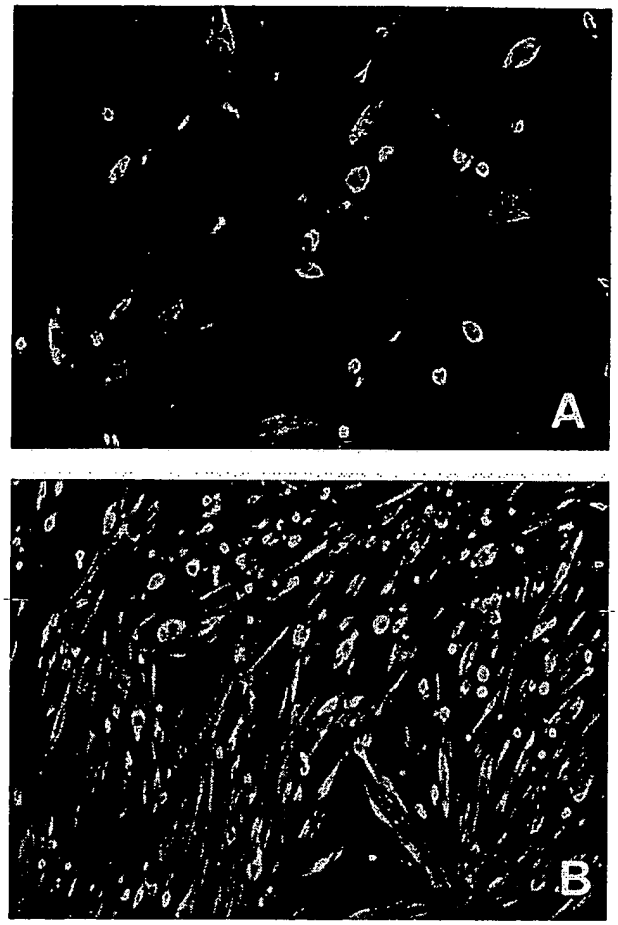

FIGURE 2. *Cultured MDCs showing immunohistochemistry staining for desmin. (A) In growth medium, the desmin-positive cells kept quiescent during first 2 to 3 days. (B) After 5 days, isolation from skeletal muscle of normal neonate mice using fusion medium in vitro, multinucleated myotubes resulting from MDC fusion are apparent. Original magnification ×200.*

conjugated to Cy3 immunofluorescence (1/200; Sigma Chemical).

Immunohistochemical Staining for Desmin

A monoclonal antibody specific for desmin was used. The cultured cells were fixed with cold methanol (−20°C) for 1 minute and blocked with 5% horse serum for 1 hour. The cells were incubated overnight at room temperature in a humid chamber with primary antibodies (1/200 monoclonal mouse anti-desmin; Sigma Chemical) in PBS, pH 7.4. After three rinses in PBS, the cells were incubated with an anti-mouse secondary antibody conjugated to Cy3 immunofluorescence (1/200; Sigma Chemical).

Immunohistochemical Staining for M-cadherin

A monoclonal antibody specific for M-cadherin was used. The cultured muscle fibers were fixed with 4% paraformaldehyde for 10 minutes and blocked with 5% horse serum for 1 hour. The cells were incubated overnight at room temperature in a humid chamber with primary antibodies (1/50 rabbit anti-mouse M-cadherin; gift from Dr. A. Wernig) in PBS, pH 7.4. After three rinses in PBS, the cells were incubated with a second antibody biotinylated anti-rabbit IgG (1/200, BA-1000, Vector). The third antibody was streptavidin Cy3 conjugate (1/200; S-6402, Sigma Chemical).

Cell Culture

Primary MDCs were plated in 35-mm collagen-coated dishes in the proliferating medium. After 24 hours, the proliferating medium was replaced by a fusion medium. The cells were maintained in the fusion medium with a daily medium change until the MDCs differentiated into myotubes.

Statistical Analysis

Values are reported as the mean ± standard deviation. The Student t test was used, and a P value less than 0.05 was considered statistically significant.

RESULTS

Positive immunohistochemical staining for M-cadherin is a marker for satellite cells. Figure 1A illustrates the appearance of a muscle fiber. The positive M-cadherin staining in Figure 1B was from a normal skeletal muscle fiber prepared from a 4-week-old normal mouse.

Different populations of MDCs were enzymatically dissociated from neonatal mouse skeletal muscle and separated by the MDCs' adhesion characteristics to collagen-coated flasks. Desmin stain-

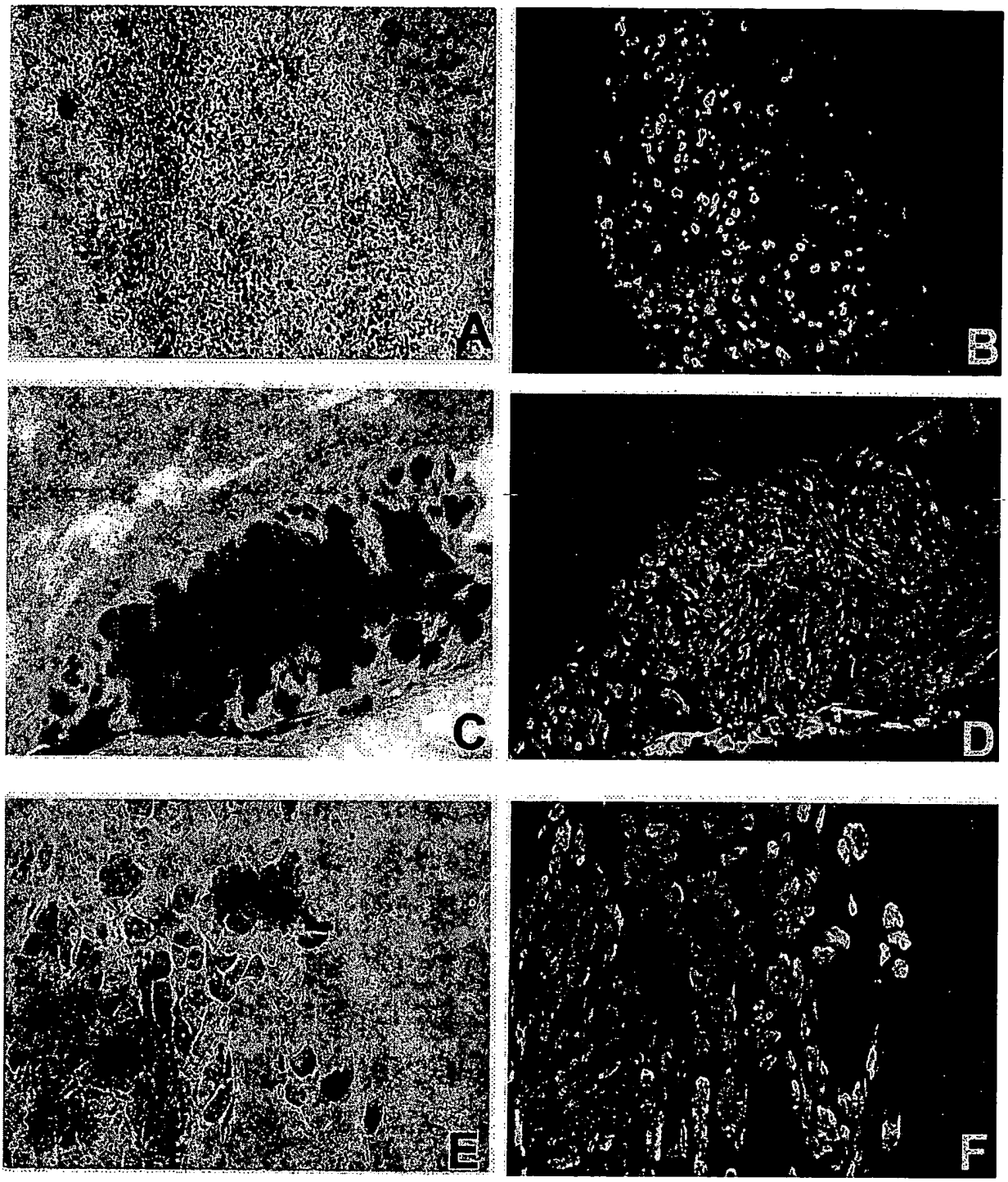

FIGURE 3. *Bladder sections showing staining of lacZ (A,C,E) and fast myosin heavy chain (B,D,F) at 5, 35, and 70 days after injection, respectively. Blue area shows lacZ-positive injected MDCs (A,C,E). Fast myosin heavy chain immunohistochemistry revealed that injected MDCs have differentiated into myotubes (B). At 35 days after injection, almost all injected MDCs have differentiated into myotubes (D). At 70 days after injection, almost all injected MDCs have differentiated into myotubes and myofibers (F). The size of the injected MDCs increased throughout the course. A and B, C and D, and E and F, respectively, show the same field with different staining. Original magnification ×200.*

ing showed that the cell cultures contained a mixture of MDCs (desmin positive), which were small and round, or spindle shaped, and fibroblasts (desmin negative), which were shaped like a branch. The cells in the later preplates included more than 90% desmin-positive cells. In growth medium, the desmin-positive cells stayed quiescent during the first 2 to 3 days after starting to isolate the cells (Fig. 2A). If left unpassaged, MDCs multiplied until they reached confluence by day 5

or 6. After the myogenic cells cease dividing, they begin to fuse and form multinucleated myotubes. Immunohistochemistry for desmin specific to striated muscle depicts skeletal MDCs after 5 days using fusion media in vitro (Fig. 2B).

Histologically, we observed a large number of MDCs expressing beta-galactosidase and fast myosin heavy chain in the bladder wall at each point (Fig. 3A-E). The injected MDCs spread between the smooth muscle layer and bladder mucosa. Many myotubes and myofibers expressing beta-galactosidase were also seen in the bladder wall at 35 and 70 days after injection (Fig. 3C,E). After injection, 1 month later, almost all MDCs had differentiated into myotubes or myofibers (Fig. 3D,F). Marking for fast myosin was weak and focal in primitive muscle cells, increasing at the myotube and myofiber stages of development.[16] At each point, fast myosin heavy chain-positive cells were found in the bladder wall (Fig. 3B,D,F). Figure 3B shows that some MDCs had already differentiated into myotubes 5 days after injection. At 35 days after injection, almost all injected MDCs had differentiated into myotubes (Fig. 3D). At 70 days after injection, almost all injected MDCs had differentiated into myotubes and myofibers (Fig. 3F). The diameters of the injected MDCs (10 cross sections chosen at random) were measured by microscope. The diameter of the injected MDCs at 5, 35, and 70 days after injection averaged 0.154 ± 0.079 mm, 0.336 ± 0.076 mm, and 0.547 ± 0.056 mm, respectively. The size of the injected MDCs increased significantly during the course of the study ($P < 0.05$).

COMMENT

In skeletal muscle, the development and regeneration of multinucleated muscle fibers start from mononucleate myoblasts of both embryos and adult animals.[17] Satellite cells, a type of undifferentiated myogenic cells, arise from somites. Satellite cells have been suggested to be muscle stem cells because of their proliferative and fusion properties.[18] Intact myofibers are surrounded by quiescent immature reserved satellite cells.[8] Satellite cells are immunohistochemically positive for M-cadherin staining.[19] We were able to demonstrate the presence of satellite cells from a normal skeletal muscle fiber prepared from a 4-week-old normal mouse.

The transplantation of MDCs in SCID mice allowed us to demonstrate the efficacy of this treatment in the absence of immunologic concerns. We used cellular transplantation into the SCID mice as a potential model for human autologous MDC transplantation. We were able to successfully isolate primary skeletal MDCs and inject them into the bladder wall. The injected MDCs led to the formation of myotubes in the smooth muscle layers of the bladder wall. We also demonstrated the feasibility of the long-term survival of MDC injection and MDC-mediated gene transfer into the bladder of immunodeficient animals for greater than 2 months.

In this study, MDCs differentiated in the bladder wall to form post-mitotic myotubes and myofibers (Fig. 3B,D,F). One of the major advantages of MDCs is the ability to fuse and become a differentiated post-mitotic cell that can persistently express a high level of the reporter gene.[20] In fact, transplanted myotubes have persisted and expressed therapeutic proteins in the brain for 6 months.[21,22]

MDCs have been extensively used as a vehicle for gene delivery in many non-muscle-related gene therapy applications, including transfer and expression of factor IX for hemophilia B[6] and systemic delivery of human growth hormone for growth retardation.[18] MDCs have also delivered the gene of human adenosine deaminase for the adenosine deaminase deficiency syndrome[23] and a muscle for muscle-related diseases such as Duchenne muscular dystrophy.[5,24] The unique advantage of MDC ex vivo gene therapy is because of MDCs' ability to become post-mitotic and create a gene reservoir of secreted molecules with a therapeutic role.

Although we are proposing the first investigation using skeletal muscle cellular myoplasty and gene transfer for repair of urinary tract smooth muscle dysfunction, we have based our work on the well-founded ground of skeletal MDC therapy for myocardial regeneration and gene therapy to the myocardium. Several recent reports using MDC transplantation for the repair of myocardial dysfunction have been published.[25–27] MDCs can differentiate into myotubes, depending on the immediately surrounding space. Since myotubes are post-mitotic, the cells will not continue to grow or spread. Therefore, the concern that MDCs would grow uncontrollably into the lower urinary tract or adjacent structures or cause bladder outlet obstruction when injected into the urethral wall is minimal. More importantly, MDCs contain satellite cells that have stem cell properties.[17] Recent investigations have demonstrated that a side population of cells was isolated from mouse muscle by fluorescence activated cell sorting (FACS) using the same method as for purification of hematopoietic stem cells. Both muscle and bone marrow side population cells share some lineage markers and have the potential to differentiate into skeletal muscle, providing evidence of the existence of bone marrow-like stem cells in muscle tissue.[28] In addition, Warejcka *et al.*[29] demonstrated the presence of pluripotent mesenchymal stem cells. These cells can be induced to differentiate into chondrocytes, osteoblasts/osteocytes, adipocytes, and

smooth muscle by culturing them in the presence of dexamethasone. The results of these investigations suggest that it is possible that MDCs, when injected into the bladder wall, may differentiate into smooth muscle cells that can augment detrusor function.

We are also investigating the capability of primary MDC transplantation to improve muscle structure and function after bladder and skeletal muscle injury. We recently observed that primary MDC transplantation enhanced muscle regeneration and muscle healing after injury.[30,31] Our results demonstrated that when MDC transplantation is done in SCID mice, the transplanted MDCs participate to enhance muscle regeneration. Although much remains to be studied and confirmed, we believe this approach has a promising and exciting future.

CONCLUSIONS

The introduction of primary skeletal MDCs into the bladder wall is feasible and results in the formation of myotubes and myofibers in the smooth muscle layers of the lower urinary tract. Primary MDC injection into the bladder demonstrated long-term persistence in SCID mice. MDC injection opens up the exciting new opportunity for treating urologic dysfunctions.

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Autologous Primary Muscle-Derived Cells Transfer into the Lower Urinary Tract

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ABSTRACT

The goal of these experiments was to establish the basic methodology for future clinical applications of muscle-derived cells (MDC) tissue engineering and gene transfer for the treatment of urological dysfunction. Primary MDC isolated via preplating techniques from adult female SD rats were transduced with retrovirus encoding the expression of β-galactosidase reporter gene. The MDC were injected into the right and left lateral walls of the bladder and proximal urethra of the autologous animals ($n = 6$) with a 10 μl Hamilton micro syringe. The amount of injected MDC ranged from 1 to 2 × 10^6 cells. The injected tissue was harvested after 7, 14, and 28 days, sectioned and examined histologically for β-galactosidase and immunohistochemically for fast myosin heavy chain specific to skeletal muscle. The tissues were also stained for anti-CD4 and anti-CD8 antibodies to assess for cellular immune reaction. We have detected a large number of autologous MDC expressing β-galactosidase and positively stained for fast myosin heavy chain in the bladder and urethral wall. Many injected myoblasts and myotubes were also seen in the bladder and urethral wall at each time point. Staining of lymphocytes with anti-CD4 and anti-CD8 antibodies was negative after MDC injection at each time point. We have demonstrated the long-term survival of autologous MDC and MDC mediated gene transfer into the bladder and urethral wall. Autologous MDC and MDC mediated gene transfer may be a promising treatment to augment bladder and urethral sphincter function.

INTRODUCTION

URINARY INCONTINENCE is a major urological health care problem in the United States. The incidence of urinary incontinence is increasing due to an aging population. Although stress urinary incontinence can occur as a result of intrinsic sphincter muscle deficiency, another common cause of urinary incontinence (urge and overflow type) is impaired detrusor contractility. Impaired contractility of smooth muscle occurs with aging, neuropathic bladder, benign prostatic hyperplasia (BPH), and diabetes mellitus. There are no effective pharmacological interventions for either condition.[1]

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In contrast to smooth muscle, skeletal muscle is constantly undergoing repair because of the presence of undifferentiated myogenic cells, known as satellite cells.[2] These cells are fusion-competent skeletal muscle precursors and, when differentiated, fuse to form myofibers capable of muscle contraction. We have previously demonstrated the ability to harvest a population of muscle-derived cells (MDC) which contain satellite cells and myoblasts from skeletal muscle biopsies.[3] We are exploring a new treatment for urinary incontinence caused by urethral and bladder dysfunction. A highly desirable method of treatment would be the use of an autologous cellular soft tissue augmentation that can bulk up and enhance deficient sphincter and impaired detrusor function.

To date, clinical applications of MDC transplantation has been in Duchenne's muscular dystrophy.[4–6] But in basic research, MDC have been used for treating the myocardial infarction model and delivering to the circulation a group of secretory non–muscle protein products, such as human growth hormone, coagulation factor IX, or erythropoietin.[7–9] MDC were used for these non–muscle-related diseases because of their ability to become postmitotic and create a gene reservoir of secreted molecules with a therapeutic role. Muscle-mediated gene delivery may be particularly advantageous for gene therapy.

The present experiment was performed to explore the feasibility and long-term survival of cellular tissue engineering using primary autologous MDC and MDC-based gene transfer into the lower urinary tract.

MATERIALS AND METHODS

Purification of muscle-derived cells

A muscle biopsy was obtained from the gastrocnemius muscle of adult female Sprague-Dawley (SD) rats under sterile conditions. The biopsied muscle was minced into a coarse slurry using razor blades. Cells were enzymatically dissociated by the addition of collagenase-type XI 0.2% for 1 h at 37°C, dispase (grade II 240 unit) for 45 min, and trypsin 0.1% for 30 min. The dissociated cells were preplated on collagen-coated flasks, and the cell population in each flask was evaluated by desmin staining, a myogenic marker. Based on previous experiments from this laboratory, the first preplated flasks contain a majority of fibroblasts while the later flasks are highly enriched with MDC. The highest enriched preplate (pp6) was used in the experiment.[3] The proliferation medium was DMEM with 10% FBS, 10% HS, 0.5% chick embryo extract, and 1% penicillin/streptomycin. The fusion medium was DMEM supplemented with 2% fetal bovine serum and 1% antibiotic solution (penicillin/streptomycin). All culture media and other supplies were purchased from Gibco Laboratories (Grand Island, NY).

Preparation of muscle-derived cells

For virus transfection, MDC were plated at a density of $1–1.5 \times 10^6$ in T 75 flasks, rinsed in Hank's balanced salt solution (HBSS, Gibco-BRL) and incubated with a retroviral vector (1×10^7 to 1×10^9 cfu/mL) suspension in 5 mL of DMEM for 4 h at 37°C. Infection with the retrovirus was performed in the presence of 8 μg/mL polybrene to improve attachment to the cultured cells. Following this initial incubation period, 10 mL of proliferation medium was added for an additional 24 h incubation at 37°C. At 24 h postinfection, the viral suspensions were removed, and the cells were rinsed with HBSS. MDC were detached using trypsin (0.25%) for 1 min, centrifuged for 5 min at 3,500 RPM and the MDC pellet was reconstituted in 20 μL of HBSS.

Retroviral vectors

The retroviral vector that we used for this study was the MFG-NB.[10] The vector contains a modified LacZ gene (nls-LacZ) that includes a nuclear-localization sequence cloned from the simian virus (SV40) large tumor antigen and is transcribed from the long terminal repeat (LTR). The titer of the viral stock was 1×10^7 to 1×10^9 (cfu/mL). The viral stock was grown in the laboratory of Dr. Johnny Huard.[11]

Injection of muscle-derived cells in animals

All experiments were performed on 6–8-week-old female SD rats in accordance with the requirements and recommendations in the Guide for the Care and the Use of Laboratory Animals (U.S. Public Health

Service, NIH publication no. 85-23, 1985). This project was approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh and the University of Pittsburgh (protocol nos. 14-98). Only certified viral free animals were used. The animals were housed in an approved viral gene therapy facility at Children's Hospital of Pittsburgh. After surgical preparation, a low midline incision was made to expose the bladder and urethra. Twenty microliters of MDC suspension in HBSS solution ($1–2 \times 10^6$ cells per 20 μL) were injected into the proximal urethra and lateral bladder wall of SD rats with a 50-μL Hamilton micro syringe. Six SD rats underwent autologous MDC injection. Two rats for each time point were evaluated.

Tissue harvest and histology

The animals were sacrificed at 7, 14, and 28 days after MDC injection, and the bladder and urethra were removed. The tissues were then snap frozen using 2-methylbutane precooled in liquid nitrogen. Analysis of the sections included hematoxylin/eosin staining and X-gal staining for the β-galactosidase reporter gene to determine the location of the gene transfer area and the viability of the injected MDC. The area around each injection site was stained, examined microscopically, and photographed.

LacZ staining by histochemical technique

The cryostat sections of the injected tissue were stained for LacZ expression as followed. They were first fixed with 1% gluteraldehyde (Sigma Chemical Co., St. Louis, MO) for 1 min and rinsed twice in phosphate-buffered saline (PBS). They were finally incubated in X-gal substrate [0.4 mg/mL 5-bromo-chloro-3-indolyl-β-D-galactoside (Boehringer-Mannheim, Indianapolis IN), 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$ in PBS overnight (37°C).

Immunohistochemical staining for fast myosin heavy chain

A monoclonal antibody specific for fast myosin heavy chain (MyHC) was used in this study. The tissues were fixed with cold acetone (−20°C) for 2 min, and they were blocked with 5% horse serum for 1 h. The sections were incubated overnight at room temperature in a humid chamber with primary antibodies [1/400 monoclonal mouse antiskeletal myosin (fast); M-4276; Sigma in PBS, at a pH of 7.4. After three rinses in PBS, the sections were incubated with an anti-mouse secondary antibody conjugated to Cy3 immunofluorescence (1/200; Sigma).

Immunohistochemical staining for CD4- and CD8-activated lymphocytes

The muscle sections were fixed with cold acetone for 10 min, and the nonspecific binding site was blocked with a mixture of goat serum (5%). The sections were incubated with avidin D blocking solution for 20 min, rinsed briefly with PBS, then incubated for 20 min with biotin blocking solution (each 4 drops per 1 mL of the diluted blocking serum; Pharmingen, San Diego, CA). The primary antibody was a mouse monoclonal antibody against CD4 and CD8 (Pharmingen) for 1 h at room temperature. Sequentially, the endogenous peroxidase activity was blocked with 1% hydrogen peroxidase for 5 min. Following several rinses in PBS, the sections were incubated with Vectastatin Elite ABC (5 mL PBS + two drops A and B; Vector) for 30 min. The peroxidase activity was revealed using 3′,3′-diaminobenzidine (1 mg/mL; Sigma) and hydrogen peroxidase (0.03%), and counterstained with hematoxylin. Muscle sections were then mounted in Gel Mount (Biomeda) and visualized by light microscopy (Nikon).

RESULTS

All animals tolerated the isolation and injection for the duration of the study without any noticeable complications.

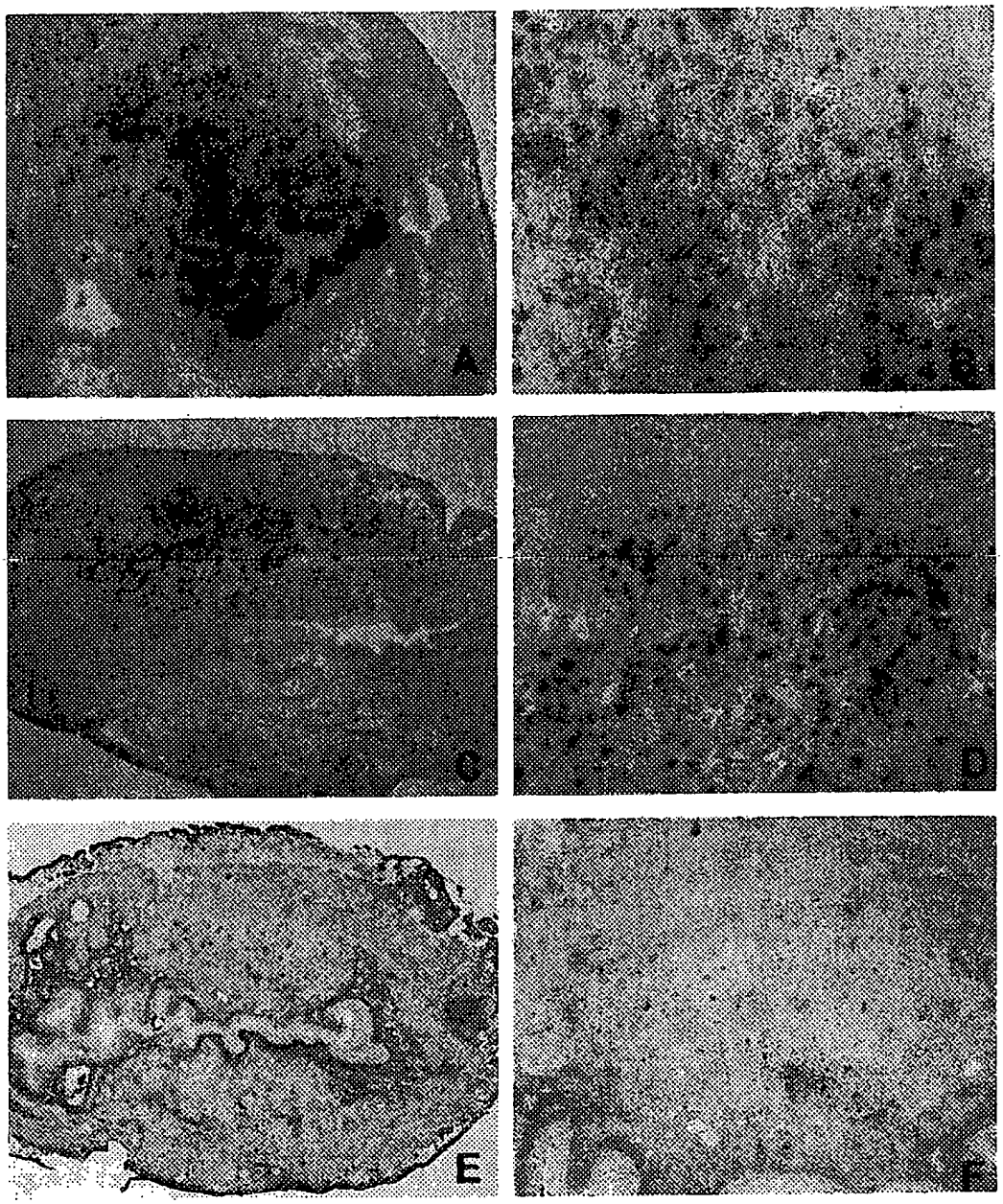

FIG. 1. Photomicrographs of bladder sections showing lac Z–positive MDC injected into the bladder walls of SD rats at 7 **(A,B)**, 14 **(C,D)**, and 28 **(E,F)** days postinjection. Note that a large number of lac Z–positive cells are observed at each time point after MDC injection. A/B, C/D, and E/F, respectively, show the same field with different magnification: ×40 (A,C,E), ×100 (B,D,F).

Assays for β-galactosidase activities

We observed a large number of cells expressing β-galactosidase in the bladder at 7 (Fig. 1A,B), 14 (Fig. 1C,D), and 28 (Fig. 1E,F) days following autologous MDC injection in rats.

We also observed a large number of cells expressing β-galactosidase in the urethral wall at 7 (Fig. 2A,B), 14 (Fig. 2C,D), and 28 (Fig. 2E,F) days following autologous MDC injection in rats. Moreover, injected MDC formed a mass that protruded toward the lumen of urethra (Fig. 2).

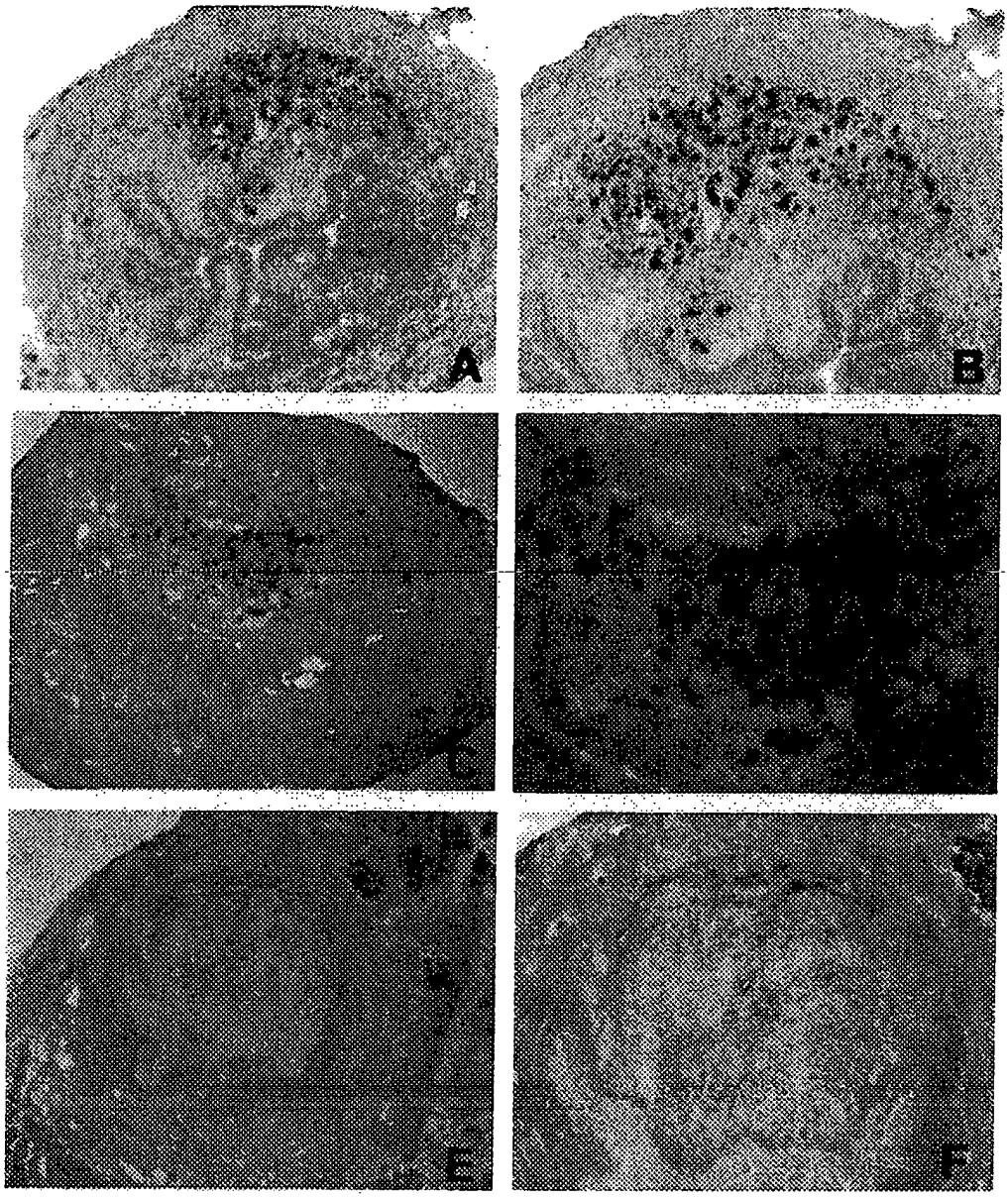

FIG. 2. Photomicrographs of urethral sections showing lac Z–positive MDC injected into the urethral walls of SD rats at 7 **(A,B)**, 14 **(C,D)**, and 28 **(E,F)** days postinjection. Note that a large number of lac Z–positive cells are observed at each time point after MDC injection. A/B, C/D, and E/F, respectively, show the same field with different magnification: ×40 (A,C,E), ×100 (B,D,F).

Although the expression gradually decreased over time, there was no evidence of severe inflammation or tissue damage such as the infiltration of inflammatory cells (e.g., platelets, macrophages, and monocytes) at the injection site (Figs. 1 and 2).

Immunochemical staining for fast myosin heavy chain

We also could observe a large number of injected MDC positively stained for fast myosin heavy chain in the bladder (Fig. 3) and urethral wall (Fig. 4) at 7 (Figs. 3A,B and 4A,B), 14 (Figs. 3C,D and 4C,D), and 28 (Figs. 3E,F and 4E,F) days following autologous MDC injection in rats.

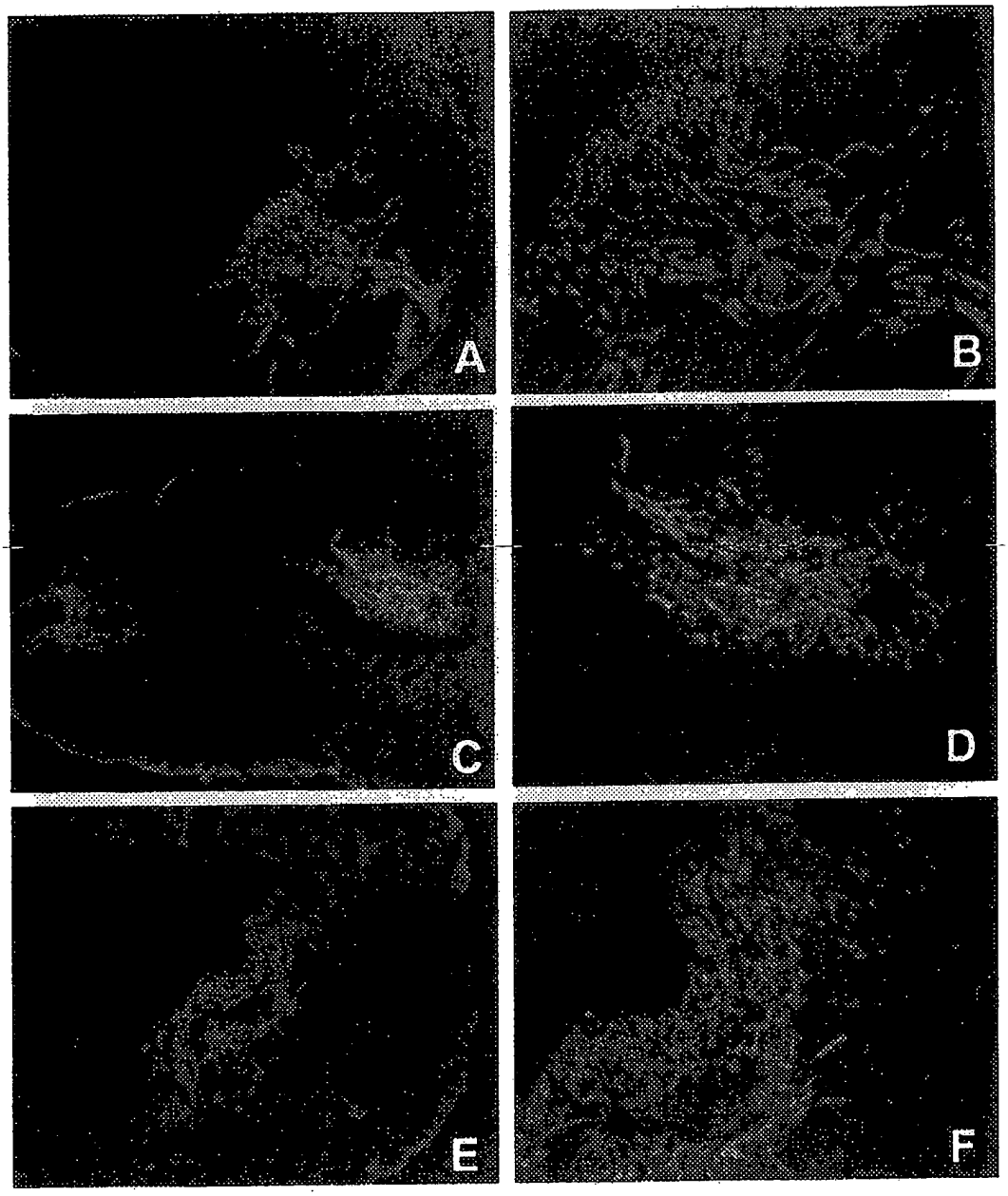

FIG. 3. Photomicrographs of bladder sections showing immunohistochemical staining for fast myosin heavy chain after MDC injection into the bladder wall of SD rats at 7 (**A,B**), 14 (**C,D**), and 28 (**E,F**) days postinjection. Note that many myotubes positively stained for fast myosin heavy chain are observed at each time point after MDC injection. A/B, C/D, and E/F, respectively, show the same field with different magnification: ×40 (A,C,E), ×100 (B,D,F).

Immunohistochemical staining for CD4- and CD8-activated lymphocytes

Positive control showed that CD4-activated lymphocytes (arrow) are identified by the browning staining after allogenic early preplated MDC injection into the bladder wall of SD rat at 28 days (Fig. 5A,B) postinjection. However, CD4- and CD8-activated lymphocytes were not observed after autologous MDC injection into the bladder wall of SD rats at any of the time points. Figures 5C,D and 5E,F illustrated negative immunohistochemical staining for CD4-activated lymphocytes at 7 and 28 days postinjection, respectively.

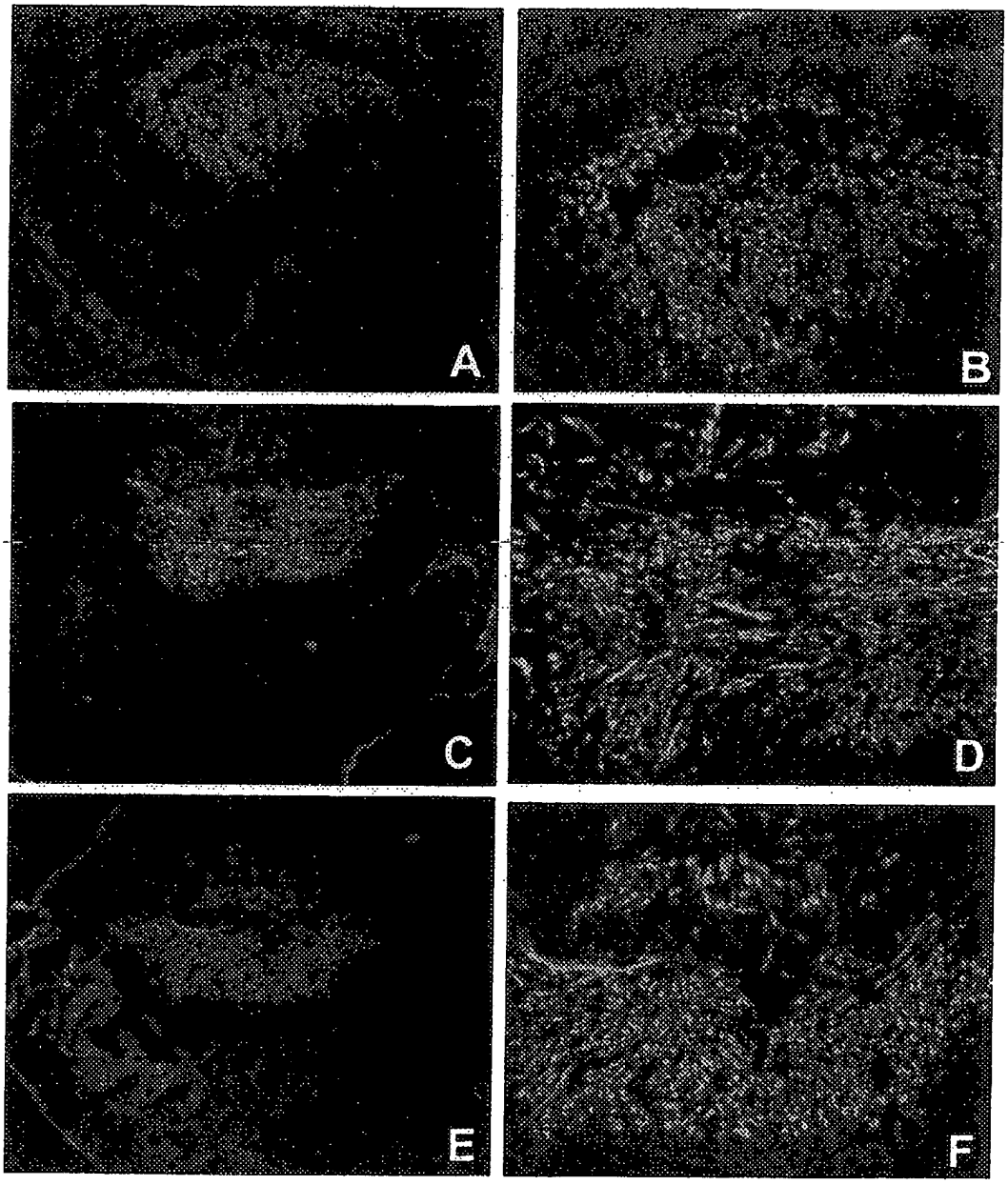

FIG. 4. Photomicrographs of urethral sections showing immunohistochemical staining for fast myosin heavy chain after MDC injection into the urethral wall of SD rats at 7 (**A,B**), 14 (**C,D**), and 28 (**E,F**) days postinjection. Note that many myotubes positively stained for fast myosin heavy chain are observed at each time point after MDC injection. A/B, C/D, and E/F, respectively, show the same field with different magnification: ×40 (A,C,E), ×100 (B,D,F).

DISCUSSION

We explored the use of autologous MDC injected into the bladder and urethral wall as a method of improving bladder contractility and treatment of stress urinary incontinence.

Adult skeletal muscle is composed of intact myofibers surrounded by a quiescent immature reserve of "satellite" cells.[2] We have recently been able to identify MDC-containing satellite cells biopsied from mouse hind limb skeletal muscle.[12]

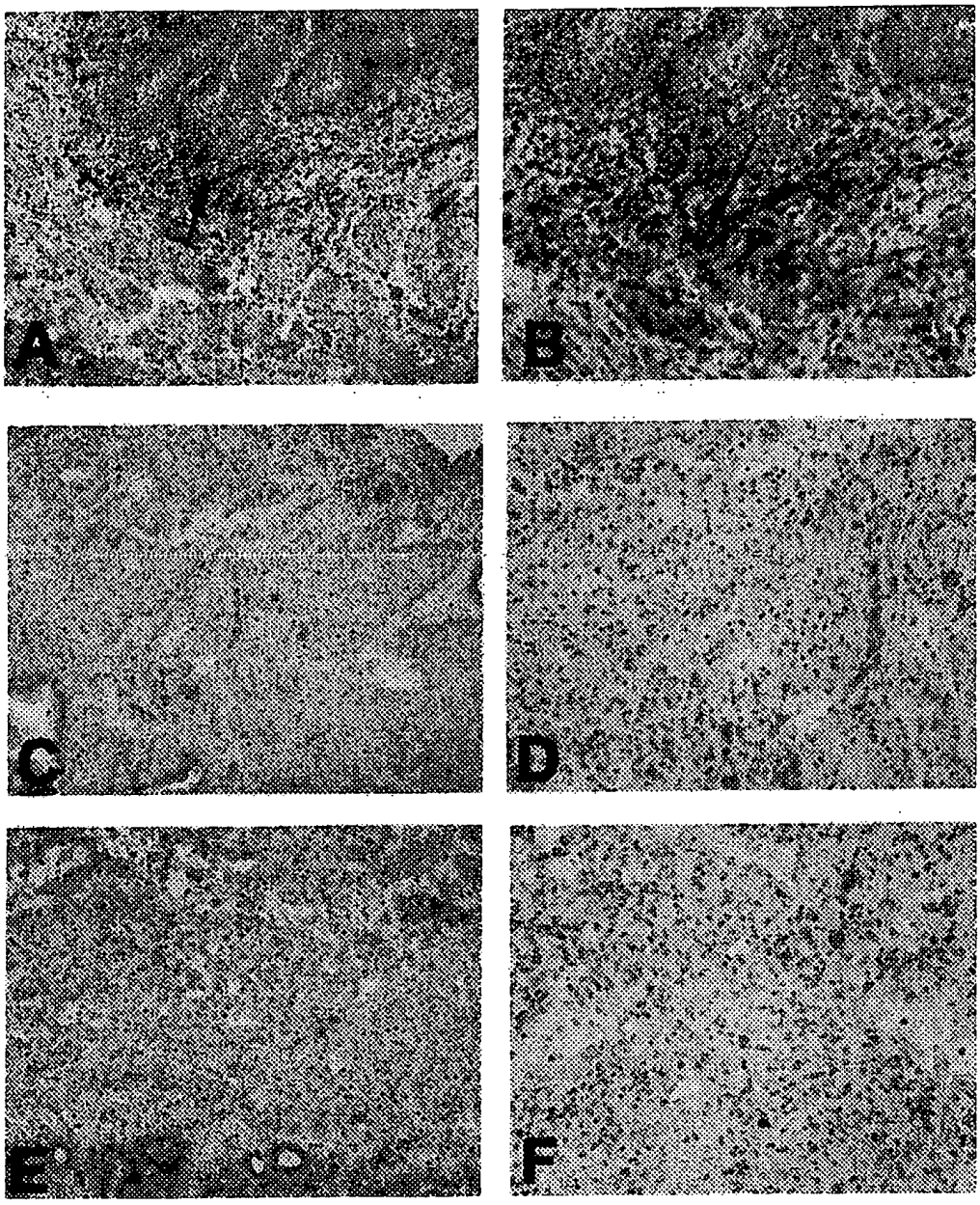

FIG. 5. Photomicrographs of bladder sections showing immunohistochemical staining for T lymphocytes. Positive control showed that CD4-activated lymphocytes (arrow) are identified by the browning staining after allogenic early preplated MDC injection into the bladder wall of SD rats at 28 days (**A,B**) postinjection. CD4-activated lymphocytes were not observed after MDC injection into the bladder wall of SD rats at 7 (**C,D**) and 28 (**E,F**) days postinjection. Original magnification, ×100 (A,C,E), ×200 (B,D,F).

We were able to successfully isolate MDC from adult animals and inject retrovirally transduced MDC into the urethral and bladder wall, resulting in the formation of myotubes in the smooth muscle layers of the bladder and urethral wall. The autologous MDC-mediated *ex vivo* gene transfer also has been investigated in our experiment using retrovirus encoding the lacZ reporter gene.

The transplantation of autologous MDC in immunocompetent adult rats allowed us to demonstrate the feasibility of this treatment without the usual immunological problems seen with allogenic transplantation.

CD4- and CD8-activated lymphocytes were not observed after autologous MDC injection into the bladder wall at any of the time points.

Although there have been many reports of tissue engineering and gene transfer in the urological field,[13–16] there have been only a few reports of injections of skeletal MDC into the urinary tract.[17,18] Following injection, MDC differentiate into myotubes and then myofibers. Since myotubes are postmitotic, the cells will not continue to grow or spread. Therefore, it seems unlikely that MDC would grow uncontrollably into the lower urinary tract or adjacent structures and thereby cause urethral outlet obstruction. This is consistent with the results of our experiments. In addition to the ability of MDC to differentiate into myotubes, MDC may also improve the function of injected organs because MDC contain satellite cells which have stem cell properties that allow differentiation into various cell types based on host environment[19] and can improve diverse tissue functions.[20] For example, the intramuscular injection of MDC engineered to secrete bone morphogenetic protein has been capable of inducing ectopic bone formation.[21] More importantly, we have observed that the MDC were in fact actively participating in bone formation *in vivo*[22] and were capable of differentiating into cartilage and bone for orthopedic repair. This last result shows that the ability of MDC to differentiate into other lineages may represent an important feature for clinical use of these cells. In addition, since MDC are capable of delivering new genes, they could be engineered to secrete many trophic substances, thus improve muscle regeneration, and enhance muscle strength, leading to improved muscle healing following injury.[23] The *ex vivo* approach using MDC allows an efficient gene transfer without apparent immunological problems associated with direct viral injection.[24] It is therefore possible to assume that autologous injections of MDC in the lower urinary tract may not only have a mass effect to increase mechanical resistance in the urethra, but also improve functional properties of the bladder and urethra if MDC can acquire the properties similar to smooth muscle fibers and/or enhance innervation of injected target organs. In future studies, we will investigate whether MDC transplantation improves muscle structure and physiological function of compromised bladder smooth muscle and sphincter muscle. Although much remains to be studied and confirmed, we believe this approach has a promising and exciting future.

In conclusion, this study demonstrated three new results in urologic tissue engineering: (1) the feasibility of autologous skeletal MDC into the lower urinary tract, (2) the long-term persistence of injected autologous MDC in the lower urinary tract, and (3) the feasibility of MDC based gene transfer to the lower urinary tract. Autologous MDC-based gene transfer and tissue engineering may be an attractive treatment alternative of stress urinary incontinence and impaired detrusor contractility.

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Results: The proportion of patients who showed a ≥ 50% reduction in the number of nightly voids during the double-blind period was significantly greater (p=<0.0001) in the group treated with drug (n=33 or 46%) than with placebo (n=5 or 7%). The number of nocturnal voids was also reduced significantly more in the drug treated group (46%) than in placebo (17%). The mean duration of the first period of sleep was also longer in the drug treated group (130 minutes) than in the placebo group (37 minutes) and 24 (33%) women treated with desmopressin (Minirin®, DDAVP®) experienced more than five hours of unbroken initial sleep per night compared with 4 (6%) in the placebo group. Nocturnal urine output also decreased significantly more in the group treated with drug (from 1.51 to 0.82 ml/min) than with placebo (from 1.44 to 1.35 ml/min). 70% of patients treated with desmopressin (Minirin®, DDAVP®) accepted an offer to continue the drug open label for a year, even though many of them had a reduction in nocturia of less than 50%.

Conclusions: Desmopressin (Minirin®,DDAVP®) is effective treatment for nocturia associated with nocturnal polyuria. It not only reduces the number of nocturnal voids and nocturnal urine volume but also improves sleep resulting in an improved quality of life as reflected in high patient acceptance for continued treatment.

1032

URETHRAL ATROPHY INCONTINENCE FOLLOWING ARTIFICIAL SPHINCTER PLACEMENT: IS CUFF DOWNSIZING EFFECTIVE?

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Introduction and Objectives: Recurrent incontinence following artificial urinary sphincter (AUS) implantation may be due to patient compliance, adverse detrusor factors, fluid leaks, erosion or urethral atrophy. This latter phenomenon occurs in approximately 6% of these patients and may occur as early as 6 months after initial implantation. Diagnosis is usually made by excluding the above factors and confirming return of intrinsic sphincteric deficiency despite a functioning device. Treatment options include: increasing reservoir pressure, proximal cuff repositioning, tandem cuff placement or cuff downsizing.This paper assesses the results of downsizing the artificial sphincter cuff to see whether this is an effective treatment option fo recurrent incontinence due to urethral atrophy.

Methods: 17 patients, ages 62-79, have undergone cuff downsizing for the management of their recurrent incontinence. Cuff downsizing was accomplished by removing the existing cuff and replacing it with a 4cm cuff within the existing 'false' capsule. Patients level of continence, and level of satisfaction, prior to and after downsizing were evaluated by validated questionnaires

Results: Urethral atrophy developed between 6 and 96 months after implantation (mean:31). Mean follow up after downsizing was 22 months (6-64). Following downsizing, pad usage and leak severity improved significantly (3.9 pads/day-0.5 pads/day; 5.4-2.1). Patient satisfaction scores improved from 15% to 85%. 16/17 patients had no complications related to the procedure, one required explantation because of urethral erosion. Continence levels in the remaining patients have been maintained.

Conclusions: All continence parameters improved dramatically after downsizing. Overall, the level of continence improved to the pre-atrophy state or better. Thus cuff downsizing is a simple and effective method of restoring continence in the presence of urethral atrophy. Despite the one case of erosion, this technique may be associated with less morbidity than other reported methods, including tandem cuff placement.

1033

NEW FUNCTIONAL SPHINCTER FORMATION AFTER ALLOGENIC MUSCLE DERIVED STEM CELL INJECTION INTO DENERVATED RAT URETHRAL SPHINCTER

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Introduction and Objectives: We determined whether muscle derived stem cells (MDSC) could survive and restore function in denervated male rat urethral sphincters.

Methods: MDSC were isolated from normal 6 weeks SD rats via preplating technique (n=6). Late preplating MDSC were first transduced with retrovirus carrying the expression of the β-galactosidase reporter gene. A midline incision was made and 1-1.5x10⁶ MDSC were injected into allogenic denervated proximal urethral sphincters. After two weeks, urethral muscle strips were prepared from normal, denervated, and denervated+MDSC injected rats. Fast twitch muscle contractions were recorded after electrical field stimulation (60V, 2.5Hz). The tissue was then sectioned, assayed for β-galactosidase activity and then counterstained with H+E.

Results: Denervated sphincters produced a decrease in fast twitch muscle contraction amplitude (5.95±3.24% of the normal sphincter contraction), while denervated+MDSC injected sphincters showed an improvement of the fast twitch muscle contraction amplitude (88.81±36.72% of the normal sphincter contraction). Histological evaluation demonstrated new skeletal muscle fiber formation at the injection site of the urethral sphincter.

Conclusions: This is the first report of restoration of deficient urethral sphincter muscle function through stem cell tissue engineering. MDSC-mediated cellular urethromyoplasty warrants further investigation as a new method to treat stress urinary incontinence.

1034

SMOOTH MUSCLE CELLS SUPPLEMENTED WITH VEGF AS A POTENTIAL TREATMENT FOR URINARY INCONTINENCE

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Introduction and Objectives: Autologous chondrocytes have been used clinically for the endoscopic treatment of urinary incontinence. Although chondrocytes can form cartilage tissue de novo, the cells may not interact in concert with native bladder muscle. We explored the feasibility of using engineered autologous bladder smooth muscle cells as a 2nd generation injectable material for urinary incontinence. These muscle cells with Vascular Endothelial Growth Factor (VEGF) would increase cell survival and enhance tissue formation.

Methods: Acellular collagen matrices, derived from porcine bladders were processed to a particle size of 100-300 um. Primary human bladder smooth muscle cells were cultured and expanded in a collagen solution at a concentration of 50x106 ml. The study included 4 groups: (I) cell-matrix suspension with VEGF(100 ng/ml); (II) cell-matrix suspension without VEGF, (III) cell only and (IV) matrix only. A 0.5 ml suspension (33% cell-matrix suspension and 67% medium) was injected subcutaneously x 4 in athymic mice. The animals were sacrificed at 1,2 and 4 weeks after injection. The retrieved tissue was analyzed grossly, histologically and immunocytochemically. Distant organs, including the kidneys, lungs and liver were analyzed for particle migration.

Results: The particle size of the collagen matrix (100-300 um) was confirmed by scanning electron microscopy. At retrieval, the cell-matrix implants retained most of their volume, compared to the cell only and matrix only groups (I: 0.3 ml, II; 0.28 ml, III; 0.14, IV; 0.18 ml). The volume of the retrieved tissue remained constant at all time points for each group. The cell-matrix suspensions supplemented with VEGF formed the largest diameters of muscle tissue (5.2 mm), compared to the cell-matrix without VEGF (4.4 mm) and the cell only group (3.0 mm). The muscle tissue formed by the VEFG supplemented group had a much higher blood vessel content per high power field when compared to the other groups. Immunocytochemical analyses using smooth muscle specific alpha-actin antibodies confirmed the cell phenotype. There was no evidence of matrix particle migration.

Conclusions: Bladder smooth muscle cells can be harvested, expanded, and easily injected in vivo, where they reconstitute into muscle tissue. Improved muscle tissue formation and volume preservation is possible with the use of collagen matrix particles as a delivery vehicle and VEGF as pro-angiogenic factor. This novel system may be potentially useful for the endoscopic treatment of urinary incontinence.

1035

TREATMENT OF LOWER URINARY TRACT DYSFUCTION AND CHRONIC PELVIC PAIN WITH BOTULINUMTOXIN A INJECTION INTO THE URETHRAL SPHINCTER MUSCLE

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Introduction and Objectives: Pelvic floor muscle dysfunction is an integral component of Lower Urinary Tract Dysfunction (LUTS) and most Chronic Pelvic Pain Syndromes (CPPS). As muscle spasticity reflects a disturbance in central nervous system regulatory pathways, it was postulated that treatment of the pelvic floor muscular dysfunction, may also help reverse the CNS disturbance causing pain.

Methods: A neuro-urological work-up of 27 patients (20 women, 7 men) with LUTS and CPPS, was completed. 200 units Botulinum Toxin Type A [BTX] were then injected into the external urethral sphincter transurethrally. Lower urinary tract function, pelvic floor dynamics and pain symptoms and were then re-evaluated four weeks later.

Results: All patients suffered from a pathological pelvic floor tenderness, an inability to consciously regulate their pelvic floor muscle, urethral hypersensitivity/ hyperalgesia and urethral muscle hyperactivity. The basic parameters of bladder function (capacity, sensitivity, compliance) were normal. Urethral sphincter pressure and residual volumes were increased. Uroflow parameters were decreased. The BTX-injection was followed by a lowering of external sphincter tone, from an abnormally high to normal ranges. There was a parallel lessening of pelvic pain and urethral hypersensitivity/ hyperalgesia of a varying degree (moderate to complete) in all patients. A BTX-related decrease of the peak urethral pressure, the post-void residual volume and an increase of [illegible] urodynamically recorded.

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IDENTIFICATION OF β-ADRENOCEPTOR SUBTYPES IN LOWER URINARY TRACT OF THE FEMALE PIG

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ABSTRACT

Purpose: We investigated the presence and functional role of β-adrenoceptor subtypes in the bladder base and proximal urethra of the female pig.

Materials and Methods: Saturation experiments were done with 7 concentrations (0.25 to 16 nM.) of [^{3}H]-dihydroalprenolol (NEN Life Science Products, Boston, Massachusetts). Competition experiments with [^{3}H]-dihydroalprenolol were performed with unlabeled antagonists (β1 selective CGP20712A, β2 selective ICI118551 and β3 selective SR59230A). In functional studies concentration-relaxation curves to the β3-agonist BRL37344 were obtained and antagonist affinities for SR59230A were determined.

Results: CGP20712A displaced [^{3}H]-dihydroalprenolol with low affinity, suggesting that β1-adrenoceptors were not present. Displacement with ICI118551 in the bladder base and urethra best fitted a 2-site model with 20% and 28% high affinity sites (β2), respectively. Displacement experiments with SR59230A in the bladder base demonstrated that 59% of binding sites had high affinity (β3). In the urethra displacement with SR59230A best fitted a 1-site model but with a pK_i of 7.2 that was intermediate between that expected for β2 and β3-adrenoceptors. In functional studies BRL37344 induced relaxation with pEC50 values of 5.5 and 8, and a maximum relaxation response relative to 30 μM. isoprenaline of 79% and 90% in the bladder base and urethra, respectively. The affinity value of SR59230A for the response to BRL37344 was 7.87 and 7.71 in the bladder base and urethra, respectively, which were intermediate between those of β2 and β3-adrenoceptors.

Conclusions: Apparently β3-adrenoceptors are the predominant β-adrenoceptor subtype present in the lower urinary tract of the pig.

KEY WORDS: bladder; swine; urinary tract; urethra; receptors, adrenergic, beta

In several species, including humans, β-adrenoceptors have been demonstrated in the bladder and urethra, [1] where they mediate relaxation. This response may be mediated via β1, β2 or β3-receptors, or a mixture of these subtypes.[2] Recently mRNA encoding for the β3-adrenoceptor was found in human detrusor as well as that encoding for β1 and β2-adrenoceptors.[3,4] Previously we have reported that β3-adrenoceptors are the predominant β-adrenoceptor subtype present in the pig bladder dome with a β2-to-β3 ratio of 1:3.[5] However, to our knowledge identification of the β-adrenoceptor subtypes in the bladder base and urethra has not been reported.

Although slight anatomical differences in pig and human urethras have been reported,[6] the pig lower urinary tract has characteristics that are physiologically and pharmacologically similar to those in humans and it is large enough to provide tissue samples from the bladder base and proximal urethra.[7–9] We determined if β3-adrenoceptors are present in the pig lower urinary tract and, if so, assessed if they are functional and mediate a relaxation. Radioligand binding experiments with [^{3}H]-dihydroalprenolol and functional experiments using a β3-adrenoceptor agonist were performed. A selective β3-adrenoceptor antagonist was also used to confirm that the selective agonist was in fact effective via the β3-adrenoceptor.

MATERIALS AND METHODS

Female pig bladders and urethras were collected from the abattoir and immediately placed into 50 mM. ice-cold (4C) tris buffer (pH 7.4). The bladder base was separated from the bladder body at the level of the ureter and the proximal urethra was separated from the bladder at the level of the bladder neck.[10] Tissue strips were cut from the bladder base and urethra, and the mucosa and serosa were removed.

Radioligand binding study. Strips of tissues were minced into small pieces using a razor blade. A crude membrane fraction was homogenized with an Ultra-Turrax homogenizer (IKA-Werke GmbH & Co., Staufen, Germany), filtered through muslin and further homogenized with a polytetrafluoroethylene homogenizer. Homogenates were centrifuged twice at 45,000 × g. for 10 minutes at 4C and the subsequent pellet was resuspended in 5 ml. tris buffer. Membranes were used immediately in radioligand assays performed in duplicate.

Saturation experiments were done using 7 concentrations (0.25 to 16 nM.) of [^{3}H]-dihydroalprenolol with a specific activity of 111.8 Ci./mmol. Samples (100 μl.) of membrane preparation were incubated at 37C for 30 minutes with a range of radioligand concentrations to obtain a saturation curve. Nonspecific binding represented [^{3}H]-dihydroalprenolol bound in the presence of 10 μM. unlabeled propranolol (Sigma Chemical Co., St. Louis, Missouri). Incubations were terminated by adding ice-cold tris buffer, followed by rapid filtration using an M30 Cell Harvester (Brandel, Gaithersburg, Maryland). After washing the filters to remove unbound radioligand the remaining radioactivity was measured in a liquid scintillation counter.

Competition binding experiments using [^{3}H]-dihydroalprenolol (1.3 nM.) with the different concentrations of unlabelled β-adrenoceptor antagonists, including the β1-adrenoceptor selective antagonist CGP20712A, β2-adrenoceptor selec-

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tive antagonist ICI118551 (Tocris Cookson, Bristol, United Kingdom) and β3-adrenoceptor selective antagonist 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol oxalate, SR59230A, which were synthesized at our laboratory, were done to determine the ratio of β-adrenoceptor subtypes present in these tissues.[5]

In vitro functional studies. Fresh tissue samples from female pigs were immediately placed in cold (4C) Krebs-bicarbonate solution composed of 118.4 mM. NaCl, 4.7 mM. KCl, 1.9 mM. $CaCl_2$, 24.9 mM. $NaHCO_3$, 1.15 mM. $MgSO_4$, 1.15 mM. KH_2PO_4 and 11.7 mM. glucose. Longitudinal strips of tissue (10 × 3 mm.) from the bladder base and transverse strips of proximal urethra (10 × 3 mm.) were cut, and the mucosa and serosa were removed. Tissues were mounted in 30 ml. organ baths containing Krebs solution, which was maintained at 37C and continuously gassed with 95% O_2 and 5% CO_2. Tissues were subjected to a resting tension of 1 gm. and allowed to equilibrate for 60 minutes, during which they were washed every 10 minutes and resting tension was adjusted. Isometric tension generated by the tissue was recorded via (Lectromed, Letchworth, United Kingdom) UF1 force transducers to a personal computer via a Cambridge Electronic Design (Cambridge Electronic Design, Cambridge, United Kingdom) interface using Chart software (GraphPad, San Diego, California).

After equilibration the tissue strips were pre-contracted with 50 mM. potassium chloride. When the contraction had stabilized, increasing concentrations of the β3-adrenoceptor selective agonist BRL37344 (Tocris Cookson) were added cumulatively in 0.5 log unit increments. In control experiments a second concentration-response curve to BRL37344 was shifted to the right 10 to 50-fold, probably because of tachyphylaxis. Thus, only 1 concentration-response curve to BRL37344 was constructed per tissue. Using separate tissues from the same animals concentration-response curves to BRL37344 were obtained in the presence of SR59230A.

In functional studies 30 μM. isoprenaline were added after determining the concentration-response curves to BRL37344 to all tissues. The relaxation response to BRL37344 is expressed as a percent of the maximum relaxation induced by isoprenaline. Agonist potency is expressed as the mean pEC50 (negative logarithm of the molar concentration of agonist resulting in 50% of the maximum response) ± SEM.

Antagonist dissociation constants (apparent K_B values) were calculated from the equation, K_B = antagonist concentration in moles/(concentration ratio − 1), where the concentration ratio is the ratio of EC50 values in the presence and absence of antagonist. In radioligand binding studies the density of receptors and K_D were calculated by nonlinear regression analysis using Prism software (GraphPad). In displacement binding experiments the same software was used to determine K_i values and the F test was used to determine goodness of fit between 1 or 2-receptor sites.[5] Student's t test was used to determine statistical analysis difference in 2 mean values with $p < 0.05$ considered statistically significant.

RESULTS

Radioligand binding study. In the saturation binding study Scatchard analysis of [^{3}H]-dihydroalprenolol binding demonstrated a single population of binding sites with a mean K_D of 1.22 ± 0.04 and 2.92 ± 0.52 nM., and a mean density of 56.5 ± 13.5 and 61.3 ± 7.86 fmol./mg. protein in the bladder base in 6 animals and proximal urethra in 7, respectively. In competition binding experiments displacement of [^{3}H]-dihydroalprenolol with the β1-selective antagonist CGP20712A (0.1 to 2 mM.) best fitted binding to a single receptor with low affinity in the bladder base and proximal urethra, suggesting that β1-receptors were not present (see table). Displacement binding with the β2-selective antagonist ICI118551 (0.1 to 1 μM.) best fitted a 2-site model in the 2 tissues with high affinity for ICI118551 (β2-adrenoceptors) at only 20% and 28% of binding sites, respectively. Displacement binding with the β3-selective antagonist SR59230A (0.1 to 1 μM.) best fitted a 2-site model in 5of 8 animals in bladder base with 59% of binding sites having a high affinity (β3-adrenoceptors). In the urethra, displacement binding with SR59230A best fitted a 1-site model in 7 of 8 animals with a pK_i that was significantly lower than that of the high affinity site of the bladder base (7.22 versus 8.45, $p < 0.01$, see table and fig. 1).

Functional studies in vitro. BRL37344 induced relaxation with a mean pEC50 of 5.5 ± 0.12 and 8.04 ± 0.05 in tissues from the bladder base and urethra in 4 animals each, respectively. Mean maximal relaxation responses relative to maximum isoprenaline relaxation were 79% ± 3.4% and 90% ± 2.7% in the bladder base and urethra, respectively. The potency (pEC50) of BRL37344 in the urethra was significantly greater than in the bladder base ($p < 0.001$). The maximum relaxation response in the urethra was significantly greater than in the bladder base ($p < 0.05$).

In the bladder base and urethra in 4 animals each (16 tissues) SR59230A (10 to 100 nM.) antagonized concentration-response curves to BRL37344 without affecting maximum responses with an apparent pK_B of 7.87 ± 0.09 and 7.72 ± 0.11, respectively. However, the Schild plots had a slope of 0.59 ± 0.26 and 0.85 ± 0.20, respectively, which was less than unity, suggesting that responses were mediated by more than 1 receptor (figs. 2 and 3).

DISCUSSION

It has been reported that β-adrenoceptors are predominantly located in the bladder dome rather than in the bladder base. Levin et al observed in a radioligand receptor binding study using [^{3}H]-dihydroalprenolol in the rabbit and dog that receptor density was greater in the bladder body than in the bladder base or urethra.[1] Previously we have reported that in the pig bladder dome [^{3}H]-dihydroalprenolol saturation curves showed a dissociation constant of 1.40 ± 0.18 nM. and a mean density of 154.4 ± 46.2 fmol./mg. protein.[5] Thus, a similar situation was found in the current study of the pig bladder, in which β-adrenoceptor density in the dome was

Competition data on [^{3}H]DHA binding in the pig lower urinary tract

Antagonists	No. Subjects/No. 2-Site Model Best Fit	Mean pK_i Affinity Site ± SEM		Mean Hill Slope ± SEM	Mean High Affinity Site Fraction ± SEM
		High	Low		
Bladder base:					
CGP20712A	4 (0)		4.17 ± 0.37	1.91 ± 0.31	
ICI118551	10 (7)	8.58 ± 0.19	5.61 ± 0.19	0.40 ± 0.13	0.20 ± 0.02
SR59230A	8 (5)	8.45 ± 0.22	5.94 ± 0.18	0.20 ± 0.03	0.59 ± 0.03
Proximal urethra:					
CGP20712A	4 (0)		4.91 ± 0.13	1.04 ± 0.10	
ICI118551	8 (6)	8.98 ± 0.20	6.409 ± 0.09	0.30 ± 0.04	0.28 ± 0.04
SR59230A	8 (1)	7.22 ± 0.14, $p < 0.01$ Vs. bladder base		0.70 ± 0.19	

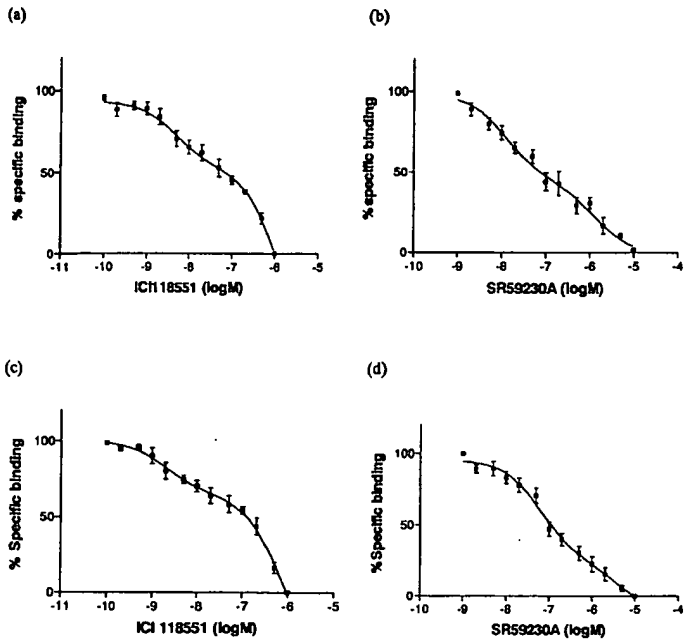

FIG. 1. Displacement of [^{3}H]-dihydroalprenolol with ICI118551 (*a*) and SR59230A (*b*) in pig bladder base (a and b) and urethra (*c* and *d*). Data represent mean ± SEM.

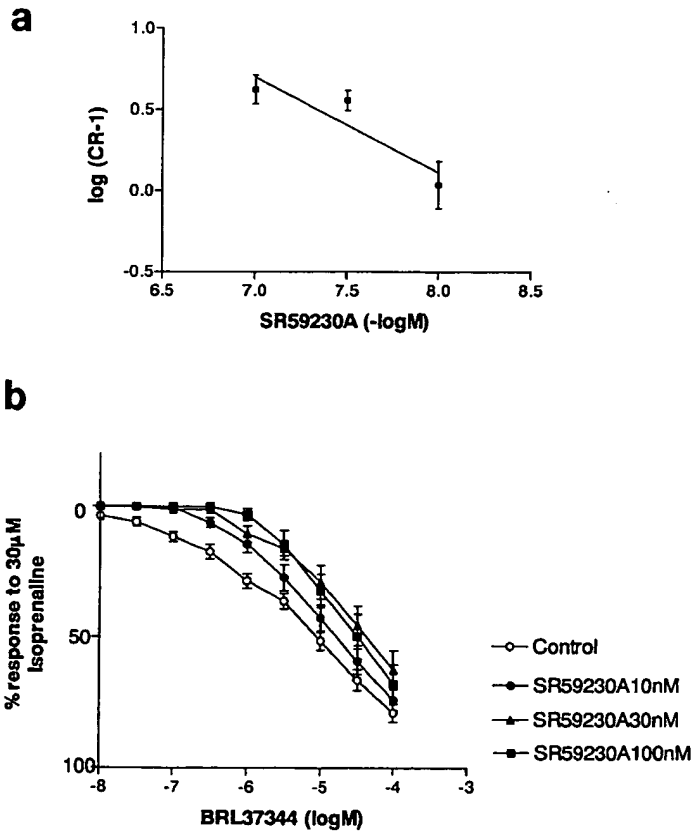

FIG. 2. Schild plot for antagonism of responses to BRL37344 by SR59230A (*a*). Effects of 10 to 100 nM. SR59230A on concentration-relaxation curves to BRL37344 in pig bladder base (*b*). Data represent mean ± SEM.

almost 3-fold greater than in the bladder base or proximal urethra. The dissociation constant of [3H]-dihydroalprenolol in the bladder base was similar to the value that we have previously obtained in the bladder dome but the affinity of radioligand in the urethra was significantly lower than in the bladder base or dome ($p < 0.05$).[5]

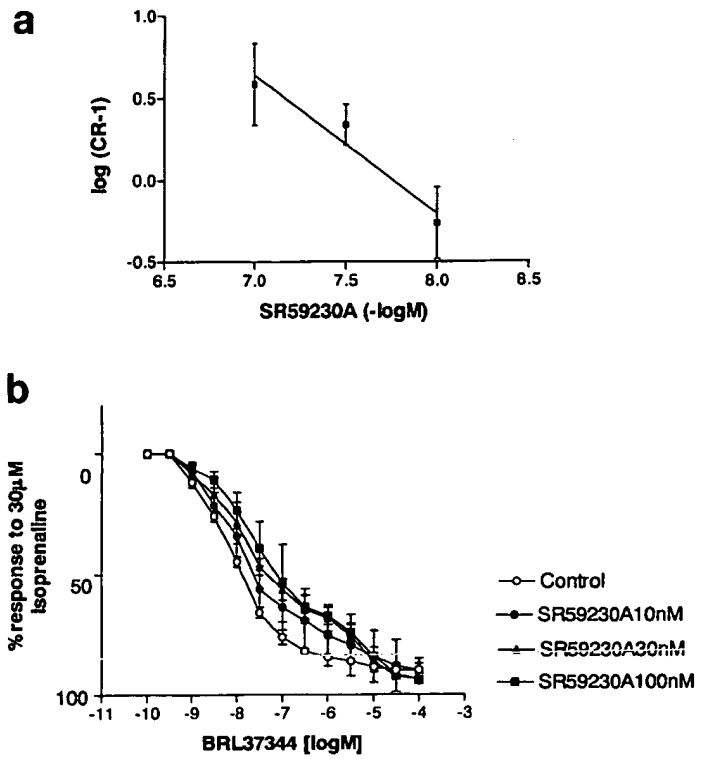

FIG. 3. Schild plot for the antagonism of responses to BRL37344 by SR59230A (*a*). Effects of 10 to 100 nM. SR59230A on concentration-relaxation curves to BRL37344 in pig proximal urethra (*b*). Data represent mean ± SEM.

In competition binding studies a predominance of β2-adrenoceptors has been reported in human and pig detrusors as well as in the pig bladder neck.[11] Recently mRNA encoding for β3-adrenoceptor was found in the bladder dome of several species, including humans. In our current series we investigated the presence of β-adrenoceptor subtypes at the protein level by radioligand binding studies using more selective β-adrenoceptor subtype antagonists, including the β3-selective antagonist SR59230A.[12]

Previously we have reported that β3-adrenoceptors are the predominant β-adrenoceptor subtypes present in the pig bladder dome with a β2-to-β3 ratio of approximately 1:3.[5] In our current study CGP20712A displaced [^{3}H]-dihydroalprenolol with a low affinity and from a single binding site in the bladder base and urethra, indicating that β1-adrenoceptors were not present in the pig lower urinary tract. Displacement with ICI118551 best fitted a 2-site model and demonstrated that the incidence of high affinity sites was 20% and 28% in the bladder base and urethra, respectively. Displacement binding with SR59230A also best fitted a 2-site model in the bladder base with high affinity (β3-adrenoceptors) at 59% of binding sites. Although displacement with SR59230A in the proximal urethra could not be fitted to a 2-site model, affinity was significantly lower than that of the high affinity sites for SR59230A (β3) in the bladder base (7.2 versus 8.5, $p < 0.01$) but significantly higher than that of the low affinity site (β2) in the bladder base (5.9, $p < 0.01$). This affinity value for the proximal urethra appears to be between that of β2 and β3-adrenoceptors compared with the reported affinity values of guinea pig trachea β2-adrenoceptors (6.6) and rat colonic β3-adrenoceptors (8.7).[12] It is also between the values previously reported for the β3 and β2-adrenoceptors of the bladder dome.[5] Furthermore the Hill slope of the competition curve to SR59230A was shallow (0.7), suggesting a mixed population of β2 and β3-adrenoceptors. Therefore, β3-adrenoceptor appears to be the major β-adrenoceptor subtype present in the

pig lower urinary tract with a β2-to[redacted]ratio of about 1:3 in all regions.

Functionally it was reported that β-adrenoceptors mediate relaxation of the detrusor muscle in several species, including humans, but it is still controversial which β-adrenoceptor subtypes have a predominant role in this relaxation.[5] It was reported that the β3-adrenoceptor has a predominant role in mediating relaxation in human bladder body,[3,4,13] although involvement of other β-adrenoceptor subtypes has also been suggested.[3,5] The β3-adrenoceptor agonist CL 316243 has also been reported to be effective for inhibiting contraction of unstable rat detrusor in vitro and in vivo.[14] In contrast, Hudman et al reported that the β2-adrenoceptor agonist clenbuterol inhibited rat detrusor muscle contraction in response to electrical field stimulation and adenosine triphosphate stimulation but not to carbachol, while clenbuterol resulted in the activation of potassium adenosine triphosphate channels with subsequent relaxation via the activation of protein kinase A.[15,16] They also reported that oral administration of clenbuterol inhibited contractile responses of detrusor muscle strips from patients with an unstable bladder but not in muscle strips from continent patients.[17]

The potency and maximum relaxation responses of BRL37344 in the bladder base (pEC50 5.5 and 79%, respectively) were comparable to those in the bladder dome in pigs and humans.[3–5,13] However, the potency of BRL37344 in the proximal urethra (pEC50 8) was significantly greater than in the bladder base, suggesting a greater involvement of β3-adrenoceptor subtypes in relaxation of the pig urethra. This result is interesting because the K_D of [^{3}H]-dihydroalprenolol in the binding study was significantly greater in the urethra than in the bladder dome[5] and base. Tate et al reported that the K_D of [^{125}I]iodocyanopindolol for pharmacologically distinct human β_3-adrenoceptors transfected for expression in Chinese hamster ovary cells (β3-CHO 0.23 ± 0.02 nM.) were greater than those for β1 or β2-adrenoceptors 0.017 ± 0.04 and 0.031 ± 0.003 nM., respectively).[18] Similar to [^{125}I]iodocyanopindolol, [^{3}H]-dihydroalprenolol may have higher binding affinity for β1 and β2-adrenoceptors than for β3-adrenoceptors. The selective β3-adrenoceptor antagonist SR59230A was used to confirm that the selective β3-agonist was in fact effective via β3-adrenoceptor. This β3-selective drug antagonized responses to BRL37344 with a high affinity of 7.9 and 7.7 in the bladder base and urethra, respectively. The Schild plots for these tissues had slopes that were significantly less than unity, indicating the involvement of more than 1 receptor. These affinity values were slightly less than those typically found for β3-adrenoceptors but greater than those for β2-adrenoceptors.[12] These affinity values were also greater than those in the human bladder body reported by Igawa et al (6.2).[3] Thus, these data suggest the involvement of β2 and β3-adrenoceptors in the bladder base and urethra, consistent with that reported for the bladder dome of the pig.[5]

The functional role of β-adrenoceptor subtypes in bladder base or urethra has not been well documented. Vaidyanathan et al reported that subcutaneous injection of the β2-adrenoceptor agonist terbutaline demonstrated a decrease in maximum urethral closure pressure in patients with neurogenic bladder dysfunction.[19] Thus, β2-adrenoceptors may have a role in facilitating bladder emptying but this relaxation must be slight and not great enough to affect bladder neck closure or urethral sealing during bladder filling in the normal bladder. Furthermore, the β2-adrenoceptor agonist clenbuterol has been reported to increase the contractility of rapidly contracting striated muscle of the pelvic floor, suggesting a possible role for these receptors in the treatment of stress incontinence.[20] It may be necessary to study the relaxation response to selective β_1- and β_2-adrenoceptor agonists and functional effects of β1 and β2-adrenoceptor antagonists to verify the role of β-adrenoceptor subtypes. Further study is ne[redacted]ary to establish the functional role of β-adrenoceptor subtypes in the bladder base and urethra.

CONCLUSIONS

Radioligand binding experiments indicate that β3-adrenoceptors appear to be the predominant β-adrenoceptor subtype present in the lower urinary tract of the female pig. Our functional data in vitro suggest that β-adrenoceptor mediated relaxation responses of the pig bladder and urethra are mediated via β2 and β3-adrenoceptors.

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Effects of the association of androgen/estrogen on the bladder and urethra of castrated rats

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Summary

Purpose: To study the effects of methyltestosterone, isolated or associated to estrogens, on the bladder and urethra of castrated adult rats.

Material & Methods: A total of 59 castrated animals were studied. They were divided into the following groups: I - placebo; II - equine conjugated estrogens; III - methyltestosterone and conjugated estrogens; IV - methyltestosterone. After 28 days of medication the animals were sacrificed and bladder and urethra cuts were obtained for the evaluation of the number of vessels, the thickness of the epithelia, and the quantity of collagen and muscular fibers.

Results: The group receiving the androgen/estrogen association presented a higher number of vessels, epithelial thickness and quantity of muscular fibers ($p < 0.05$). A smaller quantity of collagen fibers was observed in the group utilizing isolated conjugated estrogen ($p < 0.05$).

Conclusion: We concluded that the association of androgen/estrogen positively modifies important parameters in the urinary continence mechanism. Therefore, it could constitute an option for hormone replacement in postmenopausal stress urinary incontinence cases.

Key words: Androgens; Estrogens; Urinary incontinence; Collagen.

Introduction

Postmenopausal stress urinary incontinence (SUI) represents one of the main problems afflicting women, thus constituting a very important disease for its psychological and social effects.

Urinary continence is determined by the conjuction of several factors acting synergistically, such as the intraabdominal topography of the vesical neck, the urethral sphincter system, the vascular periurethral cushion, and the musculature of the pelvic and urogenital diaphragms [1]. A urethral pressure higher than the intravesical one is fundamental for urinary continence [2]. Urethral mucosa, vascularization, periurethral musculature and connective tissues are the main determinant factors of intraurethral pressure. Periurethral vascularization is responsible for a third of the urethral pressure; straited muscles are responsible for another third; and the remaining third has been attributed to the periurethral striated muscles and connective tissues [3, 4]. All these factors are highly influenced by estrogens [5, 6].

The use of hormone replacement therapy in SUI is based on the embryologic origin that is common in the genital and urinary tract [7], and also by the presence of steroid receptors in the lower urinary tract [8]. The estrogen influence on the pelvic tissues is long known [6, 9].

Lately, the estrogen/androgen association in the climacterium has earned more attention due to the better knowledge of androgen decrease after surgical or spontaneous menopause, and also, to the evidence of positive response in women presenting vasomotor symptoms and loss of libido, whenever androgen is added to the HRT [10, 11].

Because of that and also because not much is known about the androgen effects on the female lower urinary tract, we evaluated in this study, the effects of methyltestosterone, isolated or associated to equine conjugated estrogen, on the number of vessels, the epithelium thickness, and the quantity of muscular and collagen fibers in the bladder and the urethra of castrated adult rats.

Material and Methods

A total of 59 virgin adult rats (rattus norvegicus albinus) were utilized. These rats were approximately 90 days old and weighed on average 210 grams.

All the animals were confined in plastic cages with covers made out of metal bars and kept at a 22°C room temperature with fluorescent lights and a 12-hour light photo period alternated with a 12-hour dark period.

Bilateral oophorectomy was performed after the acclimatization phase through a dorsal pathway, under inhalation anesthesia with ethyl ether. Hormone administration started four days after the surgical procedure, whenever the hypoestrogenic stage was confirmed by the hormonal colpocytology. The drugs were administered by gavage over 28 uninterrupted days through metal catheters. All drugs and medicines were diluted in 0.5 ml of propylene glycol.

The animals were divided randomly into four groups:
I - constituted by 14 animals that received propylene glycol;
II - constituted by 15 rats that received a 50 µg/day dose of equine conjugated estrogen;
III - 15 rats had 0.075 mg/kg/day doses of methyltestosterone;
IV - 15 rats that received a combination of methyltestosterone and equine conjugated estrogens in the same doses above.

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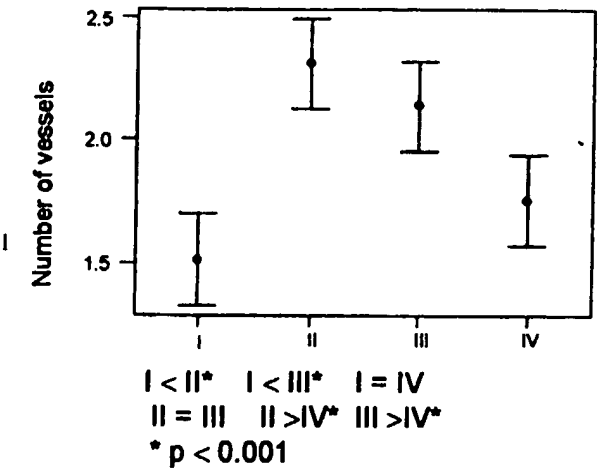


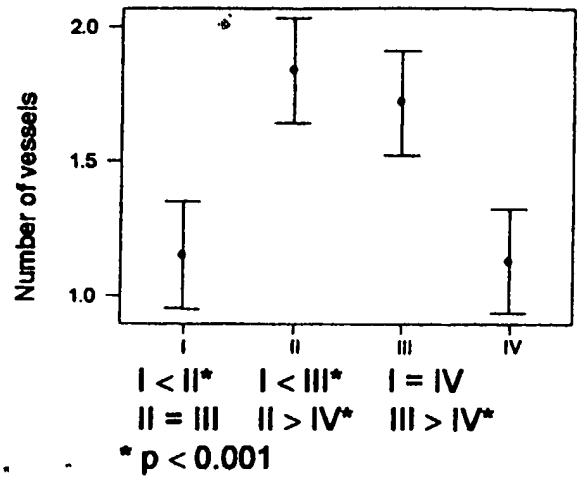


Figure 1. — Confidence intervals (γ=95%) for the number of vessels in the bladder.
Figure 2. — Confidence intervals (γ=95%) for the number of vessels in the urethra.

The animals were sacrificed with ethyl ether and, immediately afterwards, they were submitted to lower genital urinary tract removal. The material obtained was divided into two representative fragments of the bladder and the proximal urethra and fixed in formol at 10%. Following that, the pieces were dehydrated with ethyl alcohol, cleared with xylol, and included in liquid paraffin. The histologic sections were obtained in a microtome set to 5 μ and stained with hematoxylin-eosin and picrosirius red.

The morphometric analysis was performed through the image representation technique, utilizing a computerized system consisted by a microscope, a color video camera and a computer.

Ten reading fields were selected randomly for the vessel count of the proper slide. The vessels were marked with an asterisk. Epithelia thickness was determined through four linear measurements in regions randomly chosen, always in thinner areas.

A digital image amplified 400x was utilized for the muscular fibers and collagen quantification. A reticulum with 25 points geometrically distributed was attached which allowed the points occurring over the muscular and collagen fibers to be counted. Thus 20 fields were counted with a total of 500 points per animal.

Taking into consideration the nature of the studied variables, variance analysis (ANOVA) and the Tukey multiple comparisons test were utilized, and the significance level was set at 5%.

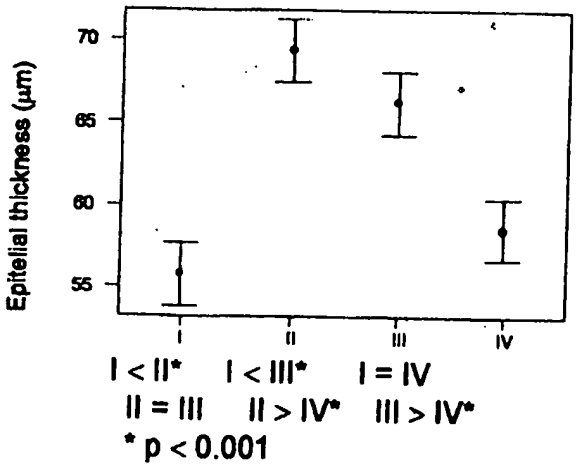
Results

A larger number of vessels was verified in the urethra as well as in the bladder among the groups receiving estrogen either isolated or associated to androgens (Figures 1 and 2).

The group utilizing the methyltestosterone isolated presented smaller thickness of the epithelium, bladder and urethra, when compared to the group receiving androgen associated to estrogen (Figures 3 and 4).

The quantity of collagen in the bladder and urethra was found to be higher in the group not receiving hormonal medication. Isolated estrogen therapy determined the lesser collagen while the groups receiving androgen were found to be similar to each other but superior to group II (Figures 5 and 6).

The number of muscular fibers in the urethra was larger in the groups utilizing hormones. In the bladder, the combination of conjugated estrogens and methyltestosterone presented the largest number of muscles (Figures 7 and 8).



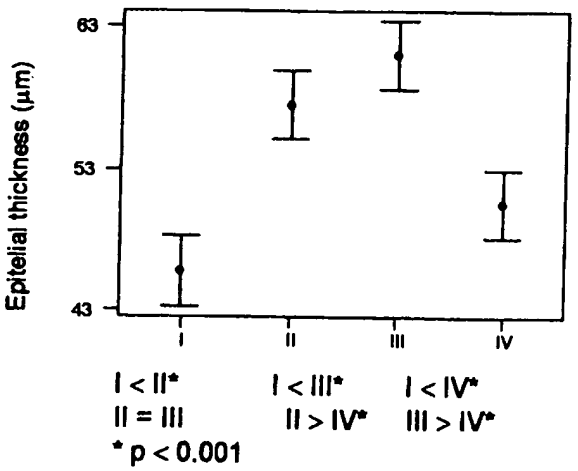


Figure 3. — Confidence intervals (γ=95%) for epithelial thickness in the bladder.
Figure 4. — Confidence intervals (γ=95%) for epithelial thickness in the urethra.

We have verified that the use of isolated estrogens promoted an important decrease of collagen among the muscular fascicles of the bladder and in the muscular layer of the urethra, as also demonstrated by other studies [18]. Regarding androgen our findings differ from those obtained by another study that observed a collagen decrease in the bladder and in the ureter of female rabbits treated with testosterone and methandrostenolone [19].

Other researches demonstrated a collagen increase in the skin of postmenopausal women treated with estradiol and testosterone [20]. There was also an increase of collagen production in women utilizing the association of estradiol and testosterone of type III, which though is more flexible [21]. Yet, androgen receptors were demonstrated in the skin fibroblasts, which suggests the susceptibility of these collagen producing cells to these hormones [22].

It is known that an abnormal depot of collagen type III in the bladder between the muscular fibers can alter detrusor contractility thus leading to urinary symtoms [23].

Our results show that the association of methyltestosterone and equine conjugated estrogens acts positively on the number of vessels, on the thickness of the epithelium, and on the number of muscular fibers in the bladder and in the urethra.

The activity of the urethra could improve with an increase in the number of vessels, the epithelium thickness, collagen, and muscular fibers since all of these parameters are involved in the maintenance of intraurethral pressure. Contrary to the bladder, it seems that urethral function is positively correlated to the amount of collagen [24].

Therefore, it seems possible to infer that the association of estrogen/androgen in postmenopause would positively affect the female lower urinary tract. These data, however, need validation in future clinical studies.

Conclusion

Our results show that the utilization of androgens associated with estrogens increased the number of vessels, the thickness of the epithelium, and the number of muscular fibers, but did not alter the amount of collagen. Therefore, the androgen/estrogen association could become one more important alternative in the therapy of postmenopausal stress urinary incontinence.

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